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## Consensus Sequences as Substrate Specificity Determinants for Protein Kinases and Protein Phosphatases

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Protein phosphorylation plays a pivotal role in the execution and regulation of many cellular functions. Consequently, phosphoproteins and the enzymes that catalyze their phosphorylation/dephosphorylation have been intensely studied. Central to our understanding of protein phosphorylation is the question of how these enzymes recognize their diverse substrate proteins. Since detailed information on substrate recognition is currently confined to those enzymes that phosphorylate/dephosphorylate serine and threonine, the remarks that follow will be confined to this group.

In 1964, Nolan and co-workers (1) observed that a chymotryptic peptide from glycogen phosphorylase could be phosphorylated by phosphorylase kinase. Later, Daile and Carnegie (2) reported that proteolytic fragments of myelin basic protein, a substrate for cAMP-dependent protein kinase (cAMP-PK),<sup>1</sup> could also serve as substrates for cAMP-PK. Bylund and Krebs (3) observed that denaturation actually transformed some proteins into substrates for cAMP-PK, while Humble and co-workers (4) demonstrated that cAMP-PK phosphorylated denatured pyruvate kinase as well as a cyanogen bromide peptide from the enzyme. The implications of these findings were that protein kinases recognized the local structure surrounding the phosphoacceptor group and that distant regions of the polypeptide chain or higher ordered protein structures play little role in determining substrate specificity. The concept rapidly evolved that the sites phosphorylated by a particular protein kinase shared a set of common sequence elements, its "consensus sequence," whose existence was necessary and sufficient for recognition by that enzyme.

In this short review we will attempt to summarize our current understanding of the role of consensus sequences in substrate recognition by protein kinases and phosphatases. While this has been the subject of several recent reviews (5-7), these works have largely focused upon the features that render synthetic peptides optimal substrates for these enzymes. Although such peptides represent powerful investigative tools, their small size and random conformation significantly limit their abilities to mimic the proteins they are intended to model. Therefore, we have focused upon the sequence features surrounding the phosphorylation sites on protein substrates. This was done both to discern patterns indicative of the existence and influence of consensus sequences in substrate recognition and to assess the degree to which the consensus sequence paradigm reliably predicts the behavior of protein kinases toward substrate proteins, both known and potential. Synthetic peptide data have been used to illuminate the significant features of those patterns revealed through the comparison of protein substrates. The limitations of space preclude

citing many of the individuals who have contributed to our understanding of protein kinase and protein phosphatase substrate specificity. We apologize for this unfortunate circumstance.

### Definition

The term consensus sequence refers to those sequence elements immediately surrounding the site(s) phosphorylated by a given protein kinase that are considered essential for its recognition and phosphorylation by that kinase. It generally takes the form of a short linear sequence of amino acids indicating the identity of the minimum set of amino acids comprising such a site and their position relative to the phosphoacceptor residue. The following assumptions are implicit in the formulation of such consensus sequences: 1) The presence of a consensus sequence on a protein is necessary and sufficient for its recognition as a substrate by a particular protein kinase. 2) The specificity-determining features of the phosphorylation site are contained in a contiguous sequence of amino acids around the phosphoacceptor. It does not include elements from different polypeptide chains or from widely scattered portions of a single polypeptide chain. 3) Not all sequence positions surrounding the phosphoacceptor group, regardless of their proximity thereto, carry equal weight in determining the recognition code.

Consensus sequences are typically represented as in the following example for p34<sup>cdc2</sup> (8): S\*/T\*-P-X-R/K. The phosphoacceptor group is denoted by an asterisk or by the letter P in parentheses. We will use the former convention and reserve the use of a P in parentheses to denote pre-existing phosphoamino acids. Where two amino acids function interchangeably, both are listed with a slash (/) separating them. Sequence positions judged to be recognition neutral are denoted by an X. This does not guarantee that all possible substitutions at these positions, or combinations thereof, will be without effect upon the properties of the resulting substrates.

### Applications and Limitations

Consensus sequences have many useful applications. As models of critical substrate recognition determinants they presumably form reflected images of the corresponding substrate binding domains. They have been used to identify autoinhibitory domains involved in the regulation of a number of protein kinases and phosphatases. They also have served as guides for the design of synthetic peptide substrates of great utility.

Much of the usefulness of the consensus sequence model lies in its simplicity. Summarizing the complexities of the substrate recognition process as sets of short recognition sequences has facilitated the evaluation and application of a large body of observations. However, it must be borne in mind that in practice the model's assumption that local primary sequence alone controls recognition represents an oversimplification. Factors such as secondary/tertiary structure or distant secondary recognition sites can and do play significant roles in substrate recognition, sometimes completely overshadowing primary sequence considerations. This can apply especially to intramolecular autophosphorylation, where the sheer physical proximity of the phosphoacceptor may drive its phosphorylation. The existence of often unknown or ill-defined negative determinants contributes further complexity to the model's application. Thus, the existence of an apparent consensus sequence does not assure that a protein can be phosphorylated, nor is it a foolproof indicator of the protein kinase responsible if phosphorylation does occur. Consensus sequence information functions best as a guide whose implications must be confirmed or refuted experimentally. Despite these limitations, the tremendous success engendered by the application of consensus sequences attests both to their practical usefulness and their genuine importance in substrate recognition.

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The abbreviations used are: cAMP-PK, cAMP-dependent protein kinase; GMP-PK, GMP-dependent protein kinase; PKC, protein kinase C; AMP-PK, AMP-activated protein kinase; CaM, calmodulin; MLCK, myosin light chain kinase; sm, smooth muscle; sk, skeletal muscle; MHCK, myosin heavy chain kinase; GSK-3, glycogen synthase kinase-3; CK, casein kinase.

## Specific Consensus Sequences

Below are discussed those protein kinases for which sufficient data exists concerning the sites they phosphorylate to provide a reasonable expectation that patterns of common features indicative of consensus sequences should be apparent, if recognition proceeds through such a mechanism. A much abbreviated summary of this information is given in Table I. Exclusion of a particular kinase does not imply that it has no consensus sequence. Unless indicated, no attempt has been made to weigh information gained from proteins known to be phosphorylated *in vivo* more heavily than that obtained *in vitro*. This has been done to secure the largest possible data base from which to make comparisons. Autophosphorylation sites were omitted for the reasons outlined earlier. To simplify discussion, the phosphoacceptor residue will be considered to be at the zero position and the adjacent N-terminal and C-terminal amino acids will be designated by the numbers  $\text{NH}_2$  ..., -3, -2, -1, 0, +1, +2, +3, ..., -COOH, etc.

**cAMP-dependent Protein Kinase**—The presence of basic amino acids, particularly arginine, N-terminal to the phosphoacceptor serine or threonine (as with most protein kinases, phosphorylation of serine is generally preferred over threonine) is a key factor in substrate recognition by cAMP-PK (9, 10). Of 93 phosphorylation sites on 52 proteins, 88 possess at least one arginine at the -2 or, more frequently (65 versus 54 cases), the -3 position. However, the optimal sequence for peptide substrates of  $\text{R-R-X-S}^*/\text{T}^*$  (10, 11) only describes 30 of these sites, the major deviations being the substitution of lysine for arginine at position -2 (14 cases) or the presence of only a single arginine in the -1 to -3 area (33 cases). It appears that *in vitro* cAMP-PK will phosphorylate sites possessing one arginine as frequently, if perhaps not as efficiently, as those with two. Surveying probable physiological targets, i.e. those thought to be phosphorylated by the enzyme in living cells, substrates having a pair of basic residues in the -1 to -3 region were favored over those with a single basic residue by nearly 2:1, indicating that cAMP-PK may be more discriminating in nature than in the laboratory, where several factors, especially the use of supraphysiological enzyme concentrations, come into play. A consensus sequence for cAMP-PK might therefore be  $\text{R-R/K-X-S}^*/\text{T}^* > \text{R-X}_2\text{-S}^*/\text{T}^* = \text{R-X-S}^*/\text{T}^*$ , which describes 95% of the sequences surveyed.

**cGMP-dependent Protein Kinase**—A survey of 16 sites phosphorylated by the cGMP-dependent protein kinase (cGMP-PK) on 10 proteins indicates the universal presence of at least one N-terminal (-1 to -4) arginine. In most cases (13/16) there are 2 or 3 basic residues present. Thus, it appears that cGMP-PK possesses a more stringent requirement for multiple basic residues than does its cAMP-regulated cousin. A possible consensus sequence for cGMP-PK might therefore be  $\text{R/K}_2\text{-X-S}^*/\text{T}^*$ , which describes 75% of the sites surveyed. Work with synthetic peptides has led to the suggestion that a +1 arginine forms the key specificity determinant for cGMP-PK (7). However, this position is so occupied in only one of the sites surveyed. Moreover, the

only physiological substrates identified to date, G-substrate (12) and the cGMP-binding phosphodiesterase (13), follow the pattern  $\text{R/K}_2\text{-X-S}^*/\text{T}^*$ , in which X + 1 is neutral.

**Protein Kinase C**—Protein kinase C (PKC) also requires basic amino acid residues near the phosphoacceptor group. PKC can be influenced by both N- and C-terminal basic residues, and avidly phosphorylates substrates containing both. A survey of 68 sites of phosphorylation on 29 proteins showed that 16 contained at least one arginine or lysine at positions -1 through -3, 19 possessed one or more at positions +1 through +3, and nearly half (31/67) were bracketed by these amino acids in the -1 through -3 and +1 through +3 regions. The positions most frequently occupied by a basic residue were +2 (34/67), -2 (31/67), -3 (25/67), and +3 (24/67). Positions -1 and +1 were so occupied in only nine and five instances, respectively.

Studies with synthetic peptides indicate that although either C- or N-terminal basic residues can serve as determinants for PKC, optimal peptides contained both (14-17). Arginine was found superior to lysine. A survey of eight proteins phosphorylated by PKC *in vivo* shows examples of sites that are double-sided (6/11), that have only C-terminal basic residues (2/11), or that have N-terminal basics only (3/11), a pattern resembling that displayed *in vitro*. A definitive consensus sequence for PKC has yet to be determined, a task complicated by potential substrate specificity differences among its isozymic forms (17). However, a summary of our current understanding might be  $(\text{R/K}_{1,2,3}\text{-S}^*/\text{T}^* \cdot (\text{X}_{2,3}, \text{R/K}_{1,3}) > \text{S}^*/\text{T}^* \cdot (\text{X}_{2,3}, \text{R/K}_{1,3}) \geq (\text{R/K}_{1,3}\text{-S}^*/\text{T}^* \cdot \text{X}_{2,3})$ .

**AMP-activated Protein Kinase**—To date six sites phosphorylated by the AMP-activated protein kinase (AMP-PK) distributed over four proteins have been sequenced (summarized in Ref. 18). Five reside on presumed physiological targets. While no clear consensus has emerged, they do share some common characteristics including a propensity for hydrophobic amino acids at positions -5 (5/6), -1 (5/6), +2 (4/6), +4 (5/6), and +5 (5/6). All possess two and often (3/6) three hydrophilic amino acids at positions -4 through -2, one of which is basic (arginine or histidine) with position -3 usually (4/6) so occupied. No acidic amino acids appear in region -1 to -6. Hydrophilic residues are generally present at positions +3 (5/6) and +6 (4/6).

**CaM Kinase II**—Exempting its behavior towards caldesmon, the multifunctional calcium/calmodulin-dependent protein kinase (CaM kinase II) is a close adherent to the consensus sequence paradigm. A survey of 16 sites phosphorylated by CaM kinase II on 12 proteins indicates that 14 possess the sequence  $\text{R-X-X-S}^*/\text{T}^*$ , a consensus originally suggested by Payne *et al.* (19) and later confirmed using synthetic peptides (20). However, when one considers caldesmon, this trend shifts markedly. CaM kinase II from smooth muscle phosphorylates eight sites on caldesmon *in vitro*, only one of which conforms to the  $\text{R-X-X-S}^*/\text{T}^*$  paradigm (21). The implications of this "aberrant" behavior have yet to be resolved.

**p34<sup>cdc2</sup>**—The best characterized of the emerging family of protein kinases involved in cell cycle control is p34<sup>cdc2</sup>. Moreno and

TABLE I  
Summary of consensus sequences most frequently recognized by protein kinases

This table represents a simplified version of the information in the text to allow rapid comparisons to be made. The reader is advised to refer to the text to gain more detailed information on the origin and predictive reliability of the sequences presented.

Protein kinase	Consensus sequence
cAMP-PK	$\text{R-R/K-X-S}^*/\text{T}^* > \text{R-X}_2\text{-S}^*/\text{T}^* = \text{R-X-S}^*/\text{T}^*$
cGMP-PK	$(\text{R/K})_2\text{-X-S}^*/\text{T}^*$
PKC	$(\text{R/K}_{1,2,3}\text{-S}^*/\text{T}^* \cdot (\text{X}_{2,3}, \text{R/K}_{1,3}) > \text{S}^*/\text{T}^* \cdot (\text{X}_{2,3}, \text{R/K}_{1,3}) \geq (\text{R/K}_{1,3}\text{-S}^*/\text{T}^* \cdot \text{X}_{2,3})$
AMP-PK	ND*
CaM kinase II	$\text{R-X-X-S}^*/\text{T}^*$
p34 <sup>cdc2</sup>	$\text{S}^*/\text{T}^* \cdot \text{P-X-R/K}^*$
Phosphorylase kinase	ND
smMLCK	$(\text{K/R}_1\text{-X})\text{-X}_1\text{-K/R}_2\text{-X}_2\text{-R-X-S}^*/\text{T}^* \cdot \text{N-V-F}$
skMLCK	$(\text{K/R}_1\text{-X})\text{-X}_1\text{-K/R}_2\text{-X}_2\text{-R-X-S}^*/\text{T}^* \cdot \text{N-V-F} > (\text{K/R}_1\text{-X})\text{-X}_1\text{-K/R}_2\text{-X}_2\text{-E-X-S}^*/\text{T}^* \cdot \text{N-V-F}$
MHCK I	$\text{R/K}_1\text{-X}_1\text{-S}^*/\text{T}^* \cdot \text{X-Y}$
MHCK II	$\text{R-G-X-S}^*/\text{T}^* \cdot \text{X-R}$
GSK-3	$\text{S}^*\text{-X-S(P)}$
CK I	$\text{S(P)}_1\text{-X}_1\text{-S}^*/\text{T}^* > (\text{D/E})_2\text{-X}_2\text{-X-S}^*/\text{T}^*$
CK II	$\text{S}^*/\text{T}^* \cdot (\text{D/E/S(P)}_1\text{-X}_{2,3})$

\* ND, unable to predict consensus sequence due to lack of sufficient information and/or influence of other factors in influencing substrate specificity.

Where X is polar.

\* Where X + 1 or -1 is a hydroxyl amino acid.

Nurse (8) surveyed eight sites phosphorylated by p34<sup>cdc2</sup> on six proteins and observed that seven conformed to the pattern S\*/T\*-P-X-R/K, with X being a polar amino acid. They noted that at least four of the six proteins become phosphorylated at the identical sites *in vivo* during mitosis.

**Phosphorylase Kinase**—Factors additional to primary sequence weigh heavily in substrate recognition by phosphorylase kinase. The evidence for this is 2-fold. First, although phosphorylase kinase will phosphorylate small peptides, such peptides fall well short of being phosphorylated with the same rate or affinity as substrate proteins. Second, cAMP-PK will phosphorylate denatured, but not native, glycogen phosphorylase at the same site as does phosphorylase kinase (3), implying that it possesses a "special" conformation.

The sites phosphorylated by phosphorylase kinase on all tissue/species forms of its major physiological target, glycogen phosphorylase, conform to the pattern K/R-R-K/R-Q-I-S\*-V/I-R-G-L (22). Synthetic peptide work indicates that the basic residues beginning at position -3 are essential for recognition (reviewed in Ref. 23). The arginine at position +2 acts as an enhancer; its removal markedly decreased the efficacy of peptide substrates. Thus, a potential consensus sequence might contain one or more basic residues between positions -3 and -5 and possibly a +2 arginine. However, other features, including the conformation of the phosphorylation site, play important roles.

**The Myosin Light Chain Kinases**—The myosin light chain kinases (MLCKs) possess an absolute specificity for myosin light chains. Comparison of the phosphorylation sites of a number of myosin light chains indicates that they conform to the following sequence pattern (reviewed in Ref. 24): (K/R)<sub>1</sub>-X<sub>1</sub>-X<sub>2</sub>-K/R<sub>1</sub>-X<sub>1</sub>-R(smooth)/E(skeletal, cardiac)-X<sub>2</sub>-S\*-N-V-F. The key difference between smooth muscle light chains *versus* those from striated (cardiac and skeletal) muscle is at position -3, where the former has arginine and the latter glutamate. Smooth muscle MLCK (smMLCK) recognizes only smooth muscle myosin light chains. Studies with synthetic peptides indicate that the three clusters of basic amino acids at -3, -6 to -10, and -11 to -14 are important recognition determinants for smMLCK, their relative influence increasing with their proximity to the phosphoacceptor serine (25). skMLCK will phosphorylate smooth, cardiac, or skeletal muscle light chains. *In vitro*, skMLCK prefers the same consensus sequence, with a -3 arginine instead of glutamate, as does smMLCK (26). This suggests that the most distant basic cluster may be an important specificity determinant under physiological conditions, even though it is not absolutely essential *in vitro*.

**Myosin I Heavy Chain Kinase**—The myosin I heavy chain kinase (MHCK I) of *Acanthamoeba castellanii* phosphorylates a single threonine or serine in the heavy chain of myosin I. The sites on three myosin I isozymes conform to the pattern R/K<sub>1,2</sub>-X<sub>1,2</sub>-S\*/T\*-X-Y, with either position +1 or -1 occupied by a hydroxyl amino acid (27). Peptide studies indicate that one N-terminal basic residue and tyrosine +2 are required for recognition, with a second N-terminal basic residue enhancing phosphorylation (28).

**Myosin II Heavy Chain Kinase**—The myosin II heavy chain kinase (MHCK II) of *A. castellanii* phosphorylates three closely clustered serine residues within the heavy chain of myosin II: T-P-S-S-R-G-G-S\*-T-R-G-A-S\*-A-R-G-A-S\*-V-R (29). The enzyme also readily phosphorylates a peptide with this sequence, indicating that it contains the information essential for recognition. These sites follow the pattern R-G-X-S\*-X-R, and this has been suggested to form the recognition determinant for MHCK II (29).

**Glycogen Synthase Kinase-3 and Synergistic Phosphorylation**—Glycogen synthase kinase-3 (GSK-3) phosphorylates glycogen synthase, cAMP-PK type II, protein phosphatase inhibitor 2, and protein phosphatase-1c, all likely physiological targets. However, phosphorylation only takes place after their prior phosphorylation by another protein kinase, a phenomenon termed "synergistic" or "hierarchical" phosphorylation. These sites possess two common characteristics, a propensity for one or more nearby (-3 to +3) proline residues, and a C-terminal location of the synergistic phosphorylation event.

Thus, the recognition determinant for GSK-3 appears to be a C-terminal serine-phosphate group. In synthetic peptides, GSK-3 recognizes the consensus sequence S\*-X<sub>1</sub>-S(P) (30). Most (7/9) sites phosphorylated by GSK-3 can conform to this model, possessing a serine residue at the +3 (one case) or +4 position that can be phosphorylated either by the "synergistic" kinase or by GSK-3 itself in an ordered mechanism. However, this synergistic phosphoserine occupies position +13 to +27 in the other two cases. Thus, proximity of a serine phosphate moiety in space, rather than along the polypeptide chain, may also confer recognition by GSK-3 (30).

**Casein Kinase I**—Casein kinase I (CK I) targets sites rich in N-terminal, negatively charged, *i.e.*, acidic or phosphorylated, amino acids. Examination of 13 sites phosphorylated by CK I on five proteins indicates that 12 possess one or more negatively charged amino acids immediately N-terminal (-1 to -5) to the phosphoacceptor group. Position -3 was so occupied 10 of 13 times, with positions -2 (7/13) and -4 (6/13) next in frequency. The residues C-terminal to the phosphoacceptor show no pattern of basic, acidic, or other character. The importance of serine phosphate as a determinant was recognized when Tuazon *et al.* (31) observed that prior dephosphorylation of casein adversely affected its phosphorylation by CK I. Flotow *et al.* (32) have since shown that a single N-terminal (-1, -2, or -3) serine or threonine phosphate residue can serve as the recognition determinant for CK I in peptides. Serine phosphate may be the more potent recognition determinant since peptides with a single serine phosphate have lower *K<sub>m</sub>* values than those with multiple glutamates or aspartates (33). This behavior suggests that phosphorylation by CK I can be regulated through a synergistic mechanism (32). The consensus sequence for CK I might be summarized as S(P)-X<sub>1,2</sub>-S\*/T\* > S(P)-X<sub>1,2</sub>-S\*/T\* > (D/E)<sub>1,2</sub>-X<sub>2,3</sub>-S\*/T\*.

**Casein Kinase II**—Casein kinase II (CK II) requires the presence of the acidic amino acid residues glutamate, aspartate, or (occasionally) serine phosphate immediately C-terminal (+1 to +3) to the phosphoacceptor serine/threonine. In synthetic peptides a single C-terminal acidic amino acid is sufficient for recognition, with the optimal position being +3 (34-36). However, in protein substrates, the presence of multiple acidic residues appears to be strongly preferred since of 37 phosphorylation sites surveyed on 19 proteins, 30 had two or more aspartic or glutamic acid residues. The distribution of these residues was 23/37 at position +1, 28/37 at +2, and 26/37 at +3. In 10 cases the close clustering of CK II sites could allow an initial phosphorylation event to introduce an additional +1 to +3 acidic determinant for the second site, three of which would have the effect of increasing the number of acidic determinants from one to two. Thus, the consensus sequence for CK II can be summarized as S\*/T\*-(D/E/S(P))<sub>1,2,3</sub>-X<sub>2,3</sub>.

### Phosphorylation Site Geometry

Early on it was recognized that geometry must help determine substrate specificity if only by denying access to potential phosphoacceptor groups. This negative "veto" role was supported by the observation that denaturation often transformed proteins into or improved them as substrates (3, 4). Small *et al.* (37) applied the Chou-Fasman secondary structure algorithm to 30 phosphorylation sites and found that 80% were predicted to exist within  $\beta$ -turns. However, spectroscopic studies of synthetic peptides (38, 39) and the use of conformationally constrained peptides (40) indicate that they bind cAMP-PK in an extended coil conformation. cGMP-PK preferred extended coil peptides as well (40). An extended conformation would be consistent with the ability to manipulate many consensus sequences as if they were linear in space. The propensity with which phosphorylation sites occur near the N and C termini of proteins is also consistent with the idea that phosphorylation sites have extended (or extendable) conformations. On the other hand, given the wide range of protein kinases and phosphoproteins extant, it seems likely that protein phosphorylation sites must exist in a variety of conformations, not just extended ones. Fiol *et al.* (30) have speculated that the frequency of proline and glycine residues proximal to GSK-3 phosphorylation sites might indicate a requirement for a  $\beta$ -turn



structure and the sites phosphorylated by the AMP-PK also occur in regions predicted to possess a high probability for forming  $\beta$ -turns (18). Certainly, for some kinases the existence of specialized, very intricate secondary/tertiary structures will no doubt form an important key to substrate recognition. The more discriminating the kinase, the more likely that the determinants recognized are conformationally complex.

### Protein Phosphatases

Since kinases and phosphatases share common protein substrates, a natural question is whether they recognize these proteins by similar mechanisms. Early on it was recognized that the number of serine/threonine-specific protein kinases far outstripped the number of serine/threonine-specific protein phosphatases. Moreover, it was observed that many protein phosphatases acted on phosphoryl groups introduced by a number of different protein kinases, indicating that protein phosphatases recognize specificity determinants different from those of protein kinases.

A frequently employed criterion for establishing that an enzyme recognizes a consensus sequence on its target(s) is determining whether it recognizes smaller fragments of substrate proteins containing the putative consensus sequence. In 1960, Graves *et al.* (41) observed that phosphorylase phosphatase would dephosphorylate, albeit slowly, a hexapeptide derived from phosphorylase  $\alpha$ . More recently, several phosphatases have been observed to dephosphorylate peptide substrates including pyruvate kinase phosphatase (42), protein phosphatase 2A (43, 44), and calcineurin (44, 45). Oftentimes the peptides that have shown promise as phosphatase substrates were much larger than those typically recognized by protein kinases. Blumenthal *et al.* (45), for example, systematically varied the length of a phosphopeptide modeled after the site on the type II regulatory subunit of cAMP-PK that is dephosphorylated by calcineurin. While a 19-residue peptide was dephosphorylated with kinetics comparable with the intact protein, decreasing the length to 15 residues simultaneously increased  $K_m$  and decreased  $V_{max}$  severalfold. Nolan *et al.* (1) reported that phosphorylase phosphatase dephosphorylated a tetradecapeptide at a 15-fold greater rate than the hexapeptide of Graves *et al.* (41). Such behavior implies that either the primary sequence "window" scanned by phosphatases is larger than that typically scanned by kinases, or that greater length is required to support the formation of higher order structures required for recognition. Attempts to resolve consensus sequences by testing peptides of varying sequence or by comparing sites on substrate proteins have revealed some general trends but have yet to yield any clearly defined consensus sequence.

In general, control of protein phosphorylation is achieved through the selective activation of individual catalysts, protein kinases, that are specifically targeted toward the appropriate substrate proteins. The requirement for a handful of phosphatases to counterbalance the activity of scores of kinases suggests the likelihood of fundamental differences in the nature of the mechanisms that control of protein phosphorylation and dephosphorylation. Among these is a much greater emphasis on mechanisms such as substrate activation, through effector binding to substrates, etc., or catalyst translocation in the control of dephosphorylation reactions (46). This emerging dichotomy in the control of protein kinase and protein phosphatase action suggests that the search for consensus sequences of the type that help provide the highly specific targeting required of many protein kinases may fail to yield similar results with regards to the protein phosphatases.

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## Emerging families of cytokines and receptors

The structure of a TNF receptor–ligand complex shows at the molecular level how members of two growing families of cytokines and receptors interact.

Not long ago, the cellular mediators known as cytokines were a loose collection of ill-defined factors with pleiotropic activities. With characteristic urgency, molecular biology has transformed the field, and cytokines are now known to form distinct clusters of functionally and genetically related proteins. The determination of the three-dimensional folds of a number of cytokines has consolidated the notion that they fall into distinct structural superfamilies. Thus, interleukin-1 (IL-1) and fibroblast growth factor (FGF) have a characteristic  $\beta$ -trefoil fold; nerve growth factor (NGF), platelet derived growth factor (PDGF) and transforming growth factor- $\beta$  (TGF- $\beta$ ) adopt 'cystine knot' folds; and the haemopoietic cytokines form four- $\alpha$ -helix bundles [1]. A parallel taxonomy is being developed for the cellular receptors that transduce the binding of cytokines into intracellular signals and that have also been found to share a small number of structural motifs [2].

As the predicted and determined structures of cytokines and their receptors can often be related to protein fold families, the conformation of any given cytokine–receptor complex should reveal general principles of the way members of these broad families interact. Thus, the structure of a complex between growth hormone and its receptor, determined by X-ray crystallography, provides an invaluable prototype for the interacting superfamilies of haemopoietic receptors and helical cytokines [2]. David Banner and colleagues have now unveiled another cytokine–receptor complex structure that promises to be equally informative — that between a tumor necrosis factor (TNF) and its receptor (TNFR) (Fig. 1) [3]. I shall describe the key features of this new structure, which provides an opportunity to review two important structural superfamilies of receptors and ligands.

### Cytokine receptor superfamily

The motif that defines this cytokine receptor superfamily was first observed in the sequence of the extracellular chain of the p75 low-affinity NGF receptor (LNGFR) as a four-fold repeat of approximately 40 amino acids (known as a Cys repeat), punctuated by six conserved cysteine residues [4]. The motif was next encountered in three receptor-like molecules with unknown ligands: OX-40, a marker of activated T cells in the rat; 4-1BB, an inducible T-cell antigen; and CD40, a B-cell antigen involved in activation by T cells [4]. Though initially christened as the 'LNGFR superfamily' [4], this receptor group is perhaps more aptly named — for reasons that will become clear as this review progresses — after two more recent entrants. These are the p55 and p75 signalling receptors, called TNFR I and TNFR II respectively, for the related cytokines TNF- $\alpha$  (also known as cachectin) and lymphotoxin (LT, also known as TNF- $\beta$ )



**Fig. 1.** Side view of the complex between lymphotoxin (LT) and the extracellular domain of TNFR-I [3]. LT binds as a trimer (of which only two subunits, the green and indigo chains, are visible in this perspective) to three receptor subunits (magenta, cyan and red ribbons), which intercalate in grooves between the LT subunits. The carboxy termini of receptor and LT chains are respectively located at the top and bottom of the complex in this view (Photograph courtesy D Banner, reproduced with permission from [3]).

[3,4]. LNGFR, which apparently has a non-signalling biological role as a cell-surface recruiter of neurotrophins, is perhaps an 'escaped' TNF ligand receptor that has evolved to bind cytokines structurally unrelated to TNF- $\alpha$  and LT promiscuously [1].

Recent additions to what I shall therefore refer to as the TNFR superfamily include Fas (or APO-1), a cell-surface antigen that can trigger apoptosis [5], CD27, an antigen identified on thymocytes and activated T cells [6] and CD30, an activation antigen from T cells that was first associated with Hodgkin's disease Reed-Sternberg cells [7]. Comparison of the sequences of human and mouse CD30 chains surprisingly suggests that, in the evolutionary lineage leading to man, there has been a relatively recent internal duplication of a segment of the CD30 gene encoding three Cys repeats [8]. In addition, a human receptor induced by lymphocyte activation is likely to be the human 4-1BB antigen [9]. A novel human TNFR homolog (TNFRh) has also been identified from a cDNA library of sequences transcribed from a

chromosome 12p fragment [10]. The closer relationship of TNFR h to TNFR I and TNFR II in their ligand-binding regions, and the fact that the TNFR h gene maps close to that encoding TNFR I [10], suggests that TNFR h might be a new receptor subtype for TNF  $\alpha$  or LT. Intriguingly, a recent paper reports the identification of an unusual TNFR-related molecule expressed in human liver cells that specifically binds TNF  $\alpha$  but not LT [11].

Animal poxviruses have been found to encode in their genomes soluble TNFR homologs that appear to subvert the host immune response by actively binding TNF cytokines. This branch of the superfamily comprises the T2 gene products of Myxoma and Shope Fibroma viruses [4], and the related G4R and crmB proteins of Variola and Cowpox viruses, respectively. A hint that this clever pathogen strategy may extend beyond viruses comes from the novel identification [12] of a primitive, fungal TNFR homolog that secreted in large amount by *Cladosporium fulvum* when it infects tomato plants. This 65 residue protein, called ECP1, is a minimal receptor that contains a single Cys repeat [13]; one can speculate that the host target of this pathogenic protein may be a protein mediator of the plant defense response.

#### Modular binding domains

The 2.9 Å resolution structure of the LT-TNFR I complex determined by Banner *et al.* [3] reveals a globular cytokine trimer engaged by three slender receptor molecules, each receptor poised alongside a narrow seam dividing adjacent LT subunits. Each receptor-ligand contact site thus represents a spatially distinct but structurally equivalent interaction. Notably, a significant portion of the 85 Å length of the crystallographically resolved receptor chain — consisting of three and a half Cys repeats — appears not to be in contact with either ligand or another receptor subunit, and clearly protrudes to the top and bottom of the bound LT trimer (Fig. 1). The opposing orientations of receptor and ligand chains also offers a general explanation of the observed mode of cross-cell binding between TNFR molecules and their membrane-tethered ligands.

The TNFR I fold (Fig. 1) confirms that the Cys repeat is an elongated protein module of approximately 30 Å length, held together by conserved C1-C2, C3-C5 and C4-C6 disulphide bonds [3]. The tandem array of Cys repeats in TNFR I has the appearance of a spiralling ladder with rungs formed by successive disulfide bridges [3]. This is reminiscent of the internal disulfide scaffolds of some toxins [14] and also of the cystine knot cytokines [1] — both, however, have protein topologies unrelated to that of the TNFR Cys repeat. The fold of an individual Cys repeat can best be described as a tandem arrangement of disulfide-tethered loops, where the C1-C2 link defines an amino-terminal loop module and an analogous C4-C6 link defines a carboxy-terminal module (Fig. 2, inset). A C3-C5 link, which is occasionally missing [4,13], then ties the stalk connecting the two loop structures to the bottom of the second loop (Fig. 2, see inset); a conserved tyrosine or phenylalanine five residues carboxy-terminal of C1 also facilitates the packing of the two loop modules [3]. In this manner, the TNFR Cys repeat can be subdivided into two smaller folding units, each of approximately 20 amino acids. This explains the puzzling

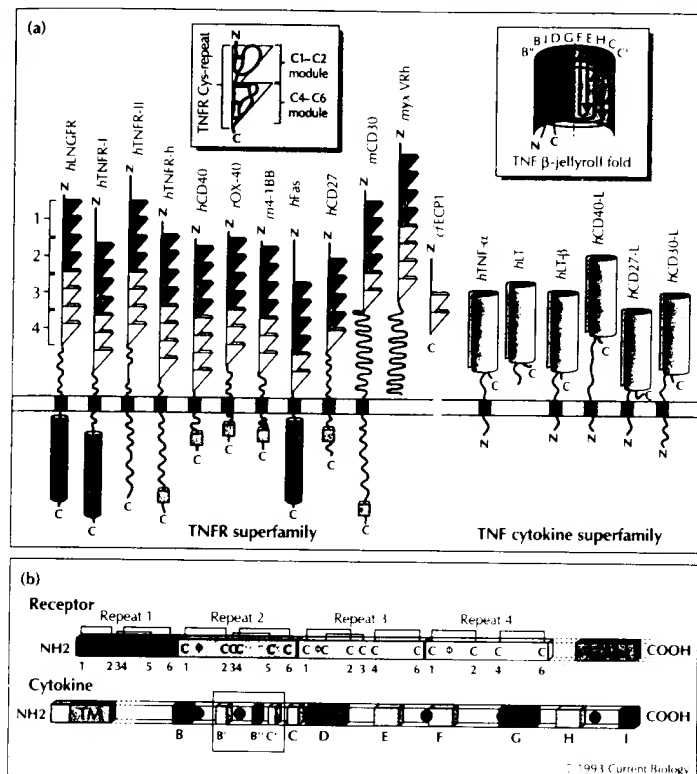
loss or truncation of exact half repeats in several superfamily receptors (repeat 4 of TNFR I and human CD30, repeat 3 of OX-40, 4 IBB and CD27; see Fig. 2). These incomplete Cys repeats are likely to contain an intact C1-C2 or C4-C6 loop module and to be able to participate in binding. This structural decomposition of the Cys repeats correlates well with exon boundaries in a composite map derived from the structures of the genes for LINGR, TNFR I, CD27, CD40, Fas and ECP1 [14].

The observation that 4 IBB binds to a variety of extracellular matrix components has led Chalupny and colleagues [15] to postulate a similarity between the modular architecture of TNFR superfamily molecules and the Cys-rich repeats of laminin-like proteins, in accord with an earlier finding of Mallet and Barclay about OX-40 [4]. The laminin Cys repeats are thought to fold as aberrant EGF repeats, with an extra disulfide link forming a tail loop [16], a topology unlike the observed fold of Cys repeats in the TNFR I crystal structure [3]. Structural studies of a nidogen binding fragment of laminin, containing three tandem repeats [16], may soon clarify the relationship of adhesive molecules to cytokine receptors.

#### Trimeric cytokines

Apart from the neurotrophins that bind LINGR, which have cystine knot folds [1,4], the known ligands of TNFRs form a distinct group of molecules homologous to TNF  $\alpha$  and LT; whereas LT is secreted, the other TNF superfamily cytokines are first produced as type II membrane proteins (Fig. 2). LT can in fact be anchored to the cell surface by forming heteromeric complexes with LT- $\beta$ , the closest relative to the founding members of the TNF superfamily [17]. In descending order of sequence similarity to TNF  $\alpha$  and LT, the next three cytokines of the TNF family to be described are the ligands of the B-cell and T-cell antigens CD40 [18], CD30 [19] and CD27 [20] (designated CD40-L, CD30-L and CD27-L, respectively). Of the ~150 amino acids that comprise the extracellular, cytokine-like domains of these molecules, only eight are absolutely conserved between all chains [13].

Crystallographic studies of TNF  $\alpha$  and LT have shown that the canonical TNF fold consists of two packed sheets of eight, antiparallel  $\beta$ -strands, arranged in a  $\beta$ -jellyroll topology with an amino-terminal insertion that contains three additional, short  $\beta$ -strands [3,21]. The  $\beta$ -jellyroll 'wedge' shapes of TNFs, with a broad base and tapering peak, is reminiscent of viral capsid proteins — indeed, Chelvanayagam and colleagues [22] have compared available  $\beta$ -jellyroll folds, including TNF  $\alpha$ , in order to define rigorous geometric, topological and packing criteria for the fold. Following a historical nomenclature labelling the strands from B to I, with B' and C' denoting consecutive strands in the inserted region, the TNF 'inner sheet' hidden in the trimer complex is formed by strands B'-B-I-D-G in correct spatial order. Similarly, the exposed 'outer' sheet is comprised of strands C'-C-H-E-F (Fig. 2, inset) [3,13,21,22]. As the elongated receptors bind in shallow grooves between cytokine subunits packed in a trimer, most of the receptor-contacting residues of the cytokine are located in the edge-located strands B and B', and strands E and F (Fig. 2, inset) — approximately 600 Å<sup>2</sup> of surface area in each of these two LT sites is buried upon receptor binding [3]. Except for



**Fig. 2.** Protein architectures of the TNFR and TNF superfamilies. (a) Shows schematically ten types of TNFRs (the prefixes give the species, as follows: h, human; m, mouse; r, rat) and two soluble receptor homologs (myx, myxoma virus; cl, *Cladosporium fulvum*). The ligand-binding regions of the receptors are divided into Cys repeats (coloured in shades from red to yellow), which can be further divided into two smaller modules (see inset). The location and nature of truncated or missing repeats is apparent from the number and color types of Cys repeats present in each receptor. The cytoplasmic domains feature two main types of motif (pink and green). The TNF cytokine superfamily consists of amino-terminally anchored transmembrane precursors (except for soluble LT), characterized by a  $\beta$ -sandwich fold (light and dark blue faces). The inset shows that the dark blue  $\beta$ -sheet is made up of strands B''-BIDG, and the light blue  $\beta$ -sheet is made up of strands C'-CHEF. (b) Shows unfolded, composite receptor and ligand chains. The upper tube shows the Cys repeats of TNFRs, with disulfide links between numbered cysteines;  $\Phi$ , conserved aromatic residues. The lower tube shows the eight  $\beta$ -strands of the cytokines, colored light or dark blue according to their  $\beta$ -sheet location; the boxed strands form the  $\beta$ -jellyroll insertion. Red circles mark the positions of cytokine residues thought to contact receptor subunits at two distinct sites in the LT-TNFR-I complex [3].

surface loop rearrangements in these regions, complexed and uncomplexed LT trimers are essentially unchanged in structure. In turn, receptor residues that contact the cytokine trimer are mostly located on discrete regions of the two central Cys repeats of the TNFR1 fold [3].

Molecular modelling of the structure of CD40-L based on the known TNF  $\alpha$  and LT protein scaffolds [23,24] has generated robust structures and provides assurance that the sparse amino acid identities between TNF superfamily cytokines are sufficient to seed a conserved three dimensional fold and quaternary assembly. The CD40-L models help to interpret the recently identified mutations of the CD40-L gene that are linked to X-linked hyper-IgM syndrome [13,24]. Point mutations and small deletions are most common: the former mostly map to predicted receptor-recognition sites in model CD40-L structures, and are thus proposed to hinder binding to CD40; chain deletions are likely to disable the protein fold by eliminating critical parts of the protein framework [14,23,24].

Before the LT-TNFR1 complex structure was known, receptor-binding sites of both TNF  $\alpha$  and LT were inferred by mapping the location of receptor blocking mutations, chemical modifications and antibody epitopes to the available trimer structures [3,13,21,23]. These studies did not seek to differentiate between the two available binding receptors for both TNF  $\alpha$  and LT, TNFR1 and TNFR2, which have quite distinct biological activities and signalling pathways. notably, TNFR1 selectively mediates

the cytotoxic activity of TNFs [25]. Seeking a heightened cytotoxic response of TNF  $\alpha$  towards malignant cells, Van Ostade *et al.* [25] latched onto an earlier observation that although human and mouse TNF  $\alpha$  bind equally well to the two human receptors, human TNF  $\alpha$  selectively binds to only mouse TNFR1 and activates the cytotoxic response. A search of available mutants revealed that a single amino acid variant (Arg32 $\rightarrow$ Trp) of human TNF  $\alpha$  produces a cytokine that is only capable of binding to human TNFR1 [26], a finding with important therapeutic potential. Guided by the new complex structure, similar protein engineering efforts for other cytokines will be greatly facilitated.

#### Signal transduction

None of the TNFR superfamily molecules has an enzymatic domain in the region of the chain extending into the cytoplasm. Instead, TNFR signalling probably relies on the oligomerization of cytoplasmic domains to unveil discrete chain motifs or create a composite tertiary epitope to attract molecules that are components of intracellular signal transduction pathways. An apparent lack of a common cytoplasmic domain shared by TNFR superfamily members has been taken as a sign that different receptors generate unrelated activation signals [4]. The present work suggests that weak but significant features of their sequences are likely to organize the receptors into three distinct classes that generate three types of signals.

The first receptor class is the recognized subgroup of LINGER, TNFR1 and Fas, which share a common cytoplasmic segment of ~200 residues [13,27], which in the case of TNFR1 and Fas is implicated in the activation of extension and apoptotic function, respectively [5,25-27]. Having rather dismissed LINGER as merely an ineffectual cytoplasmic stalk based on omotrophin signalling [13], it is difficult to discern that the homology of its cytoplasmic region to that of Fas may have important biological consequences. Rabizadeh *et al.* [28] recently reported that LINGER is, like Fas, involved in the induction of apoptosis. The interesting kink in this story is that, in contrast to TNFR1 and Fas, LINGER constitutively promotes neural cell death unless, and not when, triggered by NGF or an antibody [28].

The second receptor class is defined by the similarity of entire, but short, cytoplasmic extensions of CD27, 4-1BB, OX-40 and CD30, and regions at the carboxyterminal ends of CD30 and TNFRh [13]. In the case of 4-1BB, this domain contains a Cys Arg Cys Pro motif similar to the sequence Cys X Cys Pro found in the CD4 and CD8 cytoplasmic tails that directs binding of a Src family tyrosine kinase [9,29] — accordingly, Kim *et al.* [29] now report a direct association of 4-1BB with p56<sup>lck</sup>. This 4-1BB motif is best conserved in the CD27 chain [6]. The third group is defined by exclusion and so far contains a single member — TNFR II.

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# A Nonsecretable Cell Surface Mutant of Tumor Necrosis Factor (TNF) Kills by Cell-to-Cell Contact

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## Summary

In addition to the induction of tumor regression, tumor necrosis factor (TNF) has been implicated as the causative agent in a number of pathologies, including cachexia, septic shock, rheumatoid arthritis, autoimmunity, and induction of HIV expression. We propose that this complex physiology might be manifest by different forms of TNF: the 17 kd secretory component, the 26 kd transmembrane form, or both. To determine whether the 26 kd form of TNF was biologically active and whether its biology differed from that of the secretory component, we generated uncleavable and solely secretable mutants of TNF and studied their biological activities. We found that an uncleavable mutant of the 26 kd cell surface transmembrane form of TNF kills tumor cells and virus-infected cells by cell-to-cell contact, and that TNF need not be internalized by its target to kill. Thus, the 26 kd integral transmembrane form of TNF may function *in vivo* to kill tumor cells and other targets locally in contrast to the systemic bioactivity of the secretory component.

## Introduction

Previously we described the existence of the 26 kd cell surface transmembrane form of tumor necrosis factor (TNF), a molecule that appears to serve as a precursor to the 17 kd secretory component (Kriegler et al., 1988). This cell surface transmembrane form is present on the surface of activated monocytes (Luettig et al., 1989) as well as activated T cells (Kinkhabwala et al., 1990). We propose that the complex physiology of TNF, best characterized by its involvement in tumor regression (Old, 1985), septic shock, cachexia (Beutler et al., 1985a, 1985b; Beutler and Cerami, 1986; Tracey et al., 1986, 1987; Old, 1987; Oliff et al., 1987), autoimmunity (Held et al., 1990), and the induction of HIV expression (Rosenberg and Fauci, 1990) might reflect both the type of TNF expressed by a cell and the location and distribution of TNF-expressing cells in the body. We also suggest that activated monocytes synthesize transmembrane TNF at the site of inflammation and kill their targets by either cell-to-cell contact through the transmembrane form of TNF or local release of the TNF secretory component. In contrast, septic shock and ca-

chexia might result from acute or chronic systemic activation of monocytes, resulting in the widespread release of the TNF secretory component into the circulation of the affected individual. Furthermore, we suggest that cell-borne cytokines and cytotoxins may be the primary mediators of directed inflammatory responses. In our earlier experiments we were unable to separate the biological activities of the two forms (26 kd and 17 kd) of TNF and thus were unable to test the hypotheses proposed above.

In an attempt to separate the functions of these two TNF forms we genetically engineered mutant forms of the wild-type TNF cDNA. Mutant genes were generated that could potentially encode both uncleavable forms of 26 kd TNF and a solely secretable form of 17 kd TNF. These mutant genes were inserted into a retroviral expression vector, transfected into tissue culture cells, and subsequently analyzed.

## Results

### The 26 kd TNF Molecule Is Cleaved to the 17 kd Secretory Component at Multiple Sites

To generate uncleavable mutants of 26 kd TNF, we generated a family of mutants of the wild-type TNF gene from which we had deleted, via site-directed mutagenesis, the codons encoding the known TNF cleavage site between amino acid residues -1 and +1 of 26 kd TNF, as well as various combinations of flanking amino acids. Mutant genes were generated from which the amino acids +1 to +5, -3 to +5, and -3 to -1 were deleted (Figure 1). These mutated genes were inserted into a retroviral expression vector, PRV (a derivative of pFVXM). The resultant mutant TNF retroviral genomes were cotransfected with a  $\beta$ -actin-neo<sup>r</sup> plasmid into NIH 3T3 cells. Transfected cells were identified after selection with G418. Individual colonies were isolated and subsequently analyzed for TNF production by two criteria: first, metabolic labeling of both cell lysates and cell supernatants with [<sup>35</sup>S]cysteine followed by immunoprecipitation with anti-TNF antiserum and subsequent polyacrylamide gel electrophoresis and autoradiography; and second, analysis of TNF cytotoxicity in cell culture supernatants. Examination of the autoradiograms revealed the presence of an immunoreactive 17 kd molecule in the supernatants of cells transfected with the plasmid encoding the presumably "uncleavable" 26 kd TNF molecule (Figure 1). Similarly, media supernatants derived from such cultures were found to be positive for TNF cytotoxicity, each roughly equivalent to wild-type TNF transfectants (data not shown).

Thus, we concluded that, at least in this cell line, the 26 kd molecule is cleaved at an additional site outside of the -3 to +5 region spanning the original cleavage site. Furthermore, cleavage at this other site does not inactivate the 17 kd secretory component. To determine whether this cleavage was merely a property of NIH 3T3 cells we cotransfected Chinese hamster ovary (CHO) cells with the wild-type and mutant TNF retroviral constructions. Immu-

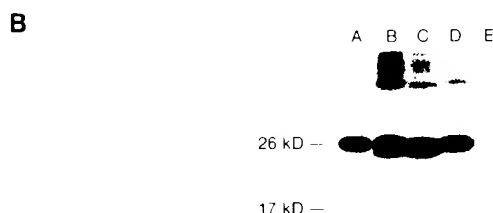
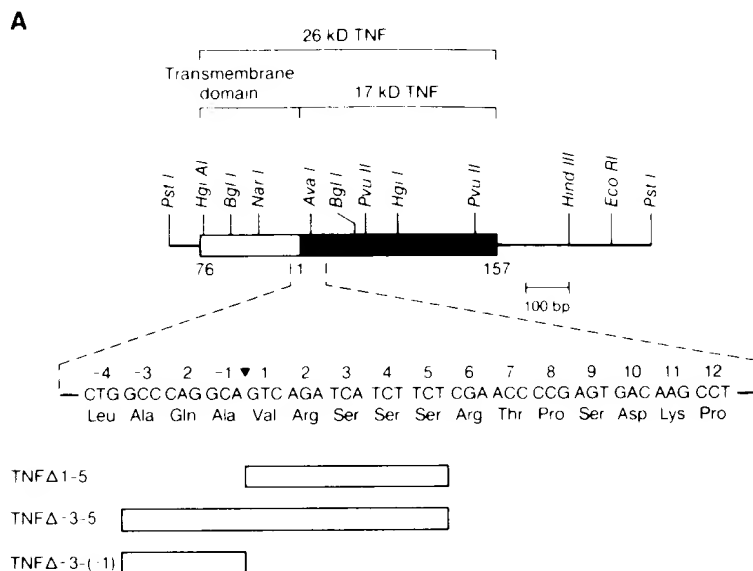


Figure 1. Schematic Depiction and Immunoprecipitation Analysis of Initial TNF Cleavage Mutants

(A) Diagram of deletion mutants flanking and spanning the 26 kD TNF cleavage site between amino acids -1 and +1 (indicated by inverted triangle).

(B) Immunoprecipitation analysis of [<sup>35</sup>S]cysteine-labeled cell lysates derived from cell lines transfected with expression plasmids encoding the cleavage mutants depicted in (A). Lane A, wild-type TNF; lane B, Δ1-5; lane C, Δ3-5; lane D, Δ3-(-1); lane E, vector control.

nonprecipitation and bioactivity analyses of the producers of these cell lines revealed a phenotype identical to that observed in NIH 3T3 cells (data not shown).

### An Uncleavable Mutant of TNF Kills Tumor Cells by Cell-to-Cell Contact

We generated additional deletion mutants of the wild-type TNF gene to determine where the alternate TNF cleavage

site was located. Deletion mutants lacking both the known cleavage site and various numbers of codons either 5' or 3' to that site were generated. These mutant genes were inserted into a retroviral expression vector and cotransfected with pβ-actin-neo into NIH 3T3 cells. Isolated G418-resistant clones were analyzed by immunoprecipitation analysis as described above. The results of this analysis indicate that deletion mutants removing the 12 or more

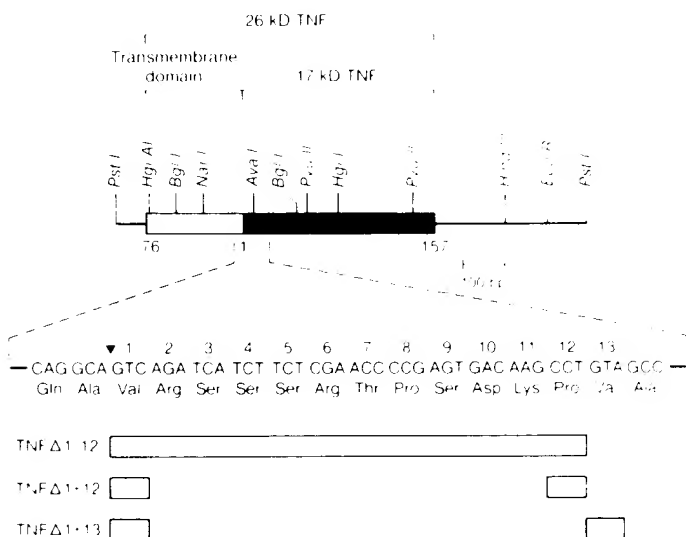


Figure 2. Schematic Depiction of TNF Mutants Lacking Both the Initial and Additional Cleavage Sites

Initial site is indicated by inverted triangle.

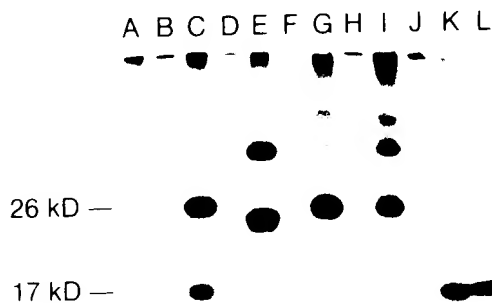


Figure 3. Immunoprecipitation Analysis of Cell Lysates and Supernatants Derived from Cell Lines Expressing TNF Cleavage Mutants Lacking Both the Initial and Additional Cleavage Sites

Isolated clones transfected with plasmid clones expressing wild-type and mutant TNF cDNAs described in Figure 2 were labeled with [<sup>35</sup>S]cysteine, lysed, immunoprecipitated, and subjected to autoradiography as described. Both cell lysates and cell supernatants are shown. Cell lysates, lanes A, C, E, G, I, and K; culture supernatants, lanes B, D, F, H, J, and L. Lanes A and B, vector control; lanes C and D, wild-type TNF; lanes E and F, Δ1-12; lanes G and H, Δ1+12; lanes I and J, Δ1+13; lanes K and L, γ SIG.

codons 3' to the known cleavage site encode TNF molecules that are not cleaved in the cytoplasm or extracellularly. Deletion of 11 or fewer codons 3' to the known cleavage site does not block cleavage in the cytoplasm and subsequent extracellular release (data not shown).

To map the fine structure of the nucleotide sequence encoding the alternate cleavage site we generated both sin-

gle codon deletion and amino acid substitution mutants flanking the known and alternate 26 kD TNF cleavage sites (Figure 2). Although amino acid substitutions at positions -1 and +1 and +12 and +13 resulted in a reduction in processing of the now-mutated forms of 26 kD TNF in the cytoplasm, only the complete deletion of amino acids +1 through +12 (Δ1-12) or amino acids +1 and +12 (Δ1+12) or +1 and +13 (Δ1+13) eliminates processing of the 26 kD molecule when cell lysates are examined. Examination of immunoprecipitates of metabolically labeled cell supernatants yields a somewhat unexpected result. Unlike the mutant Δ1-12, the mutants Δ1+12 and Δ1+13 are exclusively cleaved extracellularly. This is in striking contrast to the processing of the wild-type 26 kD TNF molecule, which is primarily cleaved in the cytoplasm of the producer cell (Figure 3). We note the presence of an immunoprecipitable band of higher molecular weight than 26 kD in lysates of cells producing TNF Δ1-12 and TNF Δ1+13. These bands are observed in all transfectant clones for these two particular mutants but not for all cleavage mutants. The origin of this additional band is unknown.

The phenotype of these uncleavable 26 kD TNF mutants is most striking. To evaluate the bioactivity of the uncleavable mutants, we employed a TNF plaque assay described previously by Kriegler et al. (1988, 1989). In this assay cells producing wild-type and mutant TNF molecules are plated and allowed to form colonies. These colonies are then overlaid with TNF-sensitive L929 cells and soft agar. After incubation for 24 hr at 37°C plaques or killing zones can be seen. Colonies producing wild-type TNF exhibit diffuse killing zones considerably larger than the

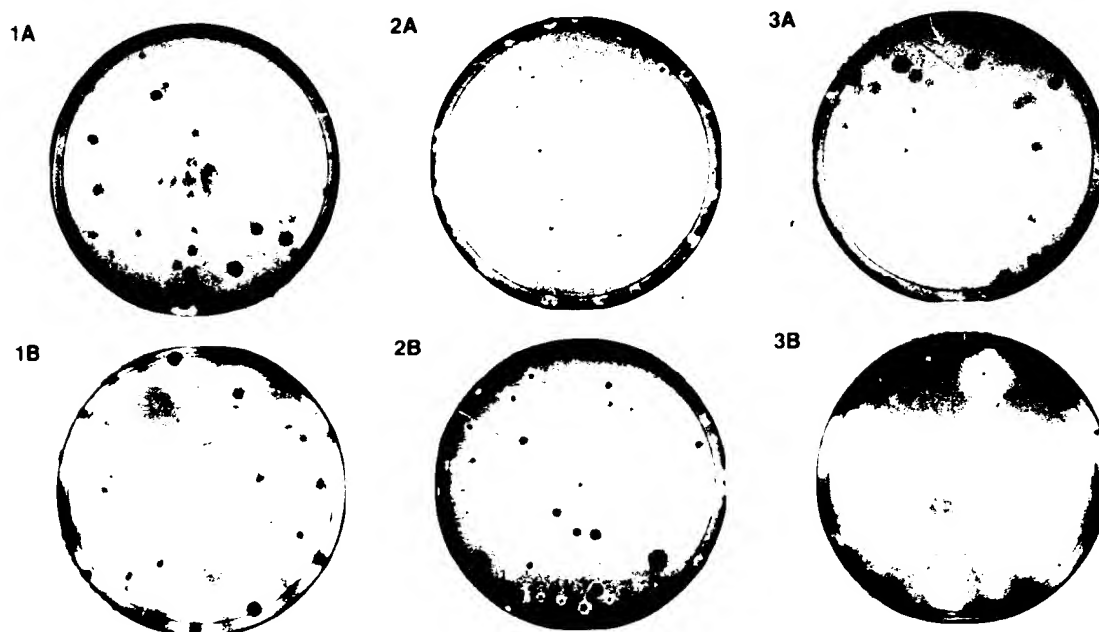


Figure 4. Plaque Assay of Wild-Type and Cleavage Mutant TNF-Expressing Cells

Plaque assays of TNF-producing clones were conducted as described in Experimental Procedures. (1A) Vector control; (1B) wild type TNF; (2A) Δ1-12; (2B) Δ1+12; (3A) Δ1+13; (3B) γ SIG.

Table 1. Analysis of TNF Mutant Cytotoxicity on Tumor Cells and Virally Infected Cells

Target	Virus	B	E:T	Effector Cells					$\gamma$ SIG
				NIH 3T3	WT	$\Delta 1-12$	$\Delta 1+12$	$\Delta 1+13$	
LM		40 $\pm$ 3	0.1:1	0 $\pm$ 2	92 $\pm$ 3	49 $\pm$ 5	18 $\pm$ 6	6 $\pm$ 2	84 $\pm$ 4
			1:1	-3 $\pm$ 3	99 $\pm$ 5	75 $\pm$ 4	62 $\pm$ 6	4 $\pm$ 3	94 $\pm$ 3
C3HA		30 $\pm$ 1	0.1:1	-2 $\pm$ 1	1 $\pm$ 0	-1 $\pm$ 1	1 $\pm$ 1	4 $\pm$ 1	2 $\pm$ 0
			1:1	-1 $\pm$ 1	2 $\pm$ 0	-2 $\pm$ 1	1 $\pm$ 1	1 $\pm$ 2	4 $\pm$ 2
C3HA	Ad5	29 $\pm$ 1	0.1:1	-4 $\pm$ 1	-3 $\pm$ 1	-1 $\pm$ 2	-5 $\pm$ 1	3 $\pm$ 4	-4 $\pm$ 1
			1:1	-1 $\pm$ 1	-1 $\pm$ 4	-4 $\pm$ 2	-2 $\pm$ 2	-2 $\pm$ 2	-3 $\pm$ 1
C3HA	d1762	45 $\pm$ 2	0.1:1	5 $\pm$ 3	53 $\pm$ 3	34 $\pm$ 7	10 $\pm$ 1	-1 $\pm$ 1	26 $\pm$ 3
			1:1	-10 $\pm$ 3	51 $\pm$ 4	60 $\pm$ 6	25 $\pm$ 4	-5 $\pm$ 3	34 $\pm$ 2

Results are expressed as the percent of specific  $^{51}\text{Cr}$  release, which equals 100 times  $^{51}\text{Cr}$  cpm released from cells incubated with effector cells minus  $^{51}\text{Cr}$  cpm released in the presence of medium alone divided by  $^{51}\text{Cr}$  cpm released in 1 N HCl (maximum release) minus  $^{51}\text{Cr}$  cpm released in medium alone. Spontaneous release of  $^{51}\text{Cr}$  by cells incubated in medium alone ranged from 29% to 45%. B, background expressed as the percentage of maximum  $^{51}\text{Cr}$  cpm released. E:T, effector:target cell ratio.

colonies themselves. This is due to the fact that the 17 kd secretory component can diffuse through the soft agar and thus manifest its bioactivity over an area larger than the colony producing the wild-type TNF (Figure 4).

When subjected to the identical assay, the TNF mutant  $\Delta 1-12$  displayed a much smaller killing zone that appeared only directly above the colony. In fact, microscopic examination of the killing zone reveals that L929 cell killing occurs only where the colony producing TNF is in direct contact with the L929 target. There is no diffusion of cytotoxicity through the agar overlay. Although the TNF mutant  $\Delta 1+12$  is cleaved extracellularly, its plaque phenotype is identical to that of the  $\Delta 1-12$  mutant, despite the fact that we observe an immunoreactive, 17 kd TNF species in the supernatant of TNF  $\Delta 1+12$  producer cells. This suggests that the 26 kd cell surface form of the molecule can kill, but when cleaved extracellularly the digestion product is no longer bioactive. An analysis of supernatants derived from these lines in a conventional L929 TNF bioassay confirms that the supernatants are negative (data not shown). The molecular structure of this mutant TNF cleavage product is under investigation.

The TNF mutant  $\Delta 1+13$  formed no plaques whatsoever when subjected to the identical plaque and conventional

TNF bioassays described above. In addition, no TNF bioactivity could be detected in the culture supernatants of mutant  $\Delta 1+13$ , although an immunoreactive 17 kd TNF species could be detected in culture supernatants (Figure 3).

To determine whether this lack of bioactivity of the TNF  $\Delta 1+13$  was due to its inability to migrate to the cell surface, wild-type TNF and mutant TNF producer cells were radioiodinated. Autoradiographic analysis of immunoprecipitated, electrophoresed products revealed that both wild-type and mutant forms of 26 kd TNF are found on the cell surface (data not shown).

#### An Uncleavable Mutant of TNF Kills Virally Infected Targets by Cell-to-Cell Contact

Additional experiments were performed to determine whether the uncleavable TNF mutants described above could kill virally infected targets as reported previously for wild-type 17 kd TNF (Gooding et al., 1988). Target murine C3HA cells are not susceptible to TNF-mediated cytotoxicity. Target C3HA cells infected with wild-type adenovirus 5 are also resistant to TNF-mediated cytotoxicity. However, C3HA cells infected with an adenovirus mutant (d1762) lacking the 14.7 kd E3 protein are sensitive to TNF-mediated cytotoxicity and as such are attractive targets for characterization

Table 2. Antibody Neutralization of TNF Mutant Cytotoxicity on Tumor Cells and Virally Infected Cells

Target	Virus	Ab	DILN	B	E:T	Effector Cells			
						$\gamma$ SIG	$\Delta 1-12$	$\Delta 1+12$	WT
LM		None		47 $\pm$ 3	1:1	94 $\pm$ 3	86 $\pm$ 2	59 $\pm$ 1	90 $\pm$ 7
		NRS	1/40	43 $\pm$ 3	1:1	82 $\pm$ 2	70 $\pm$ 5	53 $\pm$ 3	73 $\pm$ 6
		Anti-TNF	1/40	47 $\pm$ 3	1:1	-5 $\pm$ 1	5 $\pm$ 2	12 $\pm$ 3	4 $\pm$ 1
C3HA	d1762	None		48 $\pm$ 2	1:1	66 $\pm$ 4	44 $\pm$ 3	34 $\pm$ 2	37 $\pm$ 2
		NRS	1/40	48 $\pm$ 2	1:1	57 $\pm$ 2	47 $\pm$ 3	40 $\pm$ 2	25 $\pm$ 1
		Anti-TNF	1/40	48 $\pm$ 2	1:1	-3 $\pm$ 2	-2 $\pm$ 1	-2 $\pm$ 1	-4 $\pm$ 1

Results are expressed as the percent of specific  $^{51}\text{Cr}$  release, which equals 100 times  $^{51}\text{Cr}$  cpm released from cells incubated with effector cells minus  $^{51}\text{Cr}$  cpm released in the presence of medium alone divided by  $^{51}\text{Cr}$  cpm released in 1 N HCl (maximum release) minus  $^{51}\text{Cr}$  cpm released in medium alone. Spontaneous release of  $^{51}\text{Cr}$  by cells incubated in medium alone ranged from 43% to 48%. Ab, antibody. B, background expressed as the percentage of maximum  $^{51}\text{Cr}$  cpm released. E:T, effector:target cell ratio.

of the bioactivity of the cleavage mutant on a virally infected target.

The uninfected and infected cell lines described above were labeled with  $^{51}\text{Cr}$  and incubated in the presence of wild-type and mutant TNF producer cell lines. Post incubation, the culture supernatants were analyzed for chromium release (Table 1). The results of these coinubation experiments demonstrate that wild-type, mutant  $\Delta 1-12$ , and mutant  $\Delta 1+12$  are cytotoxic and mutant  $\Delta 1+13$  is not, and that cytotoxicity is generally dependent on effector:target cell ratio. This observation is consistent with the tumor cell cytotoxicity data above (Figure 4 and Table 1). Furthermore, the cytotoxicity reported in Table 1 can be neutralized with anti-TNF antiserum (Table 2), demonstrating that the cytotoxicity is TNF dependent.

#### TNF Need Not Be Internalized by the Target Cell to Kill

To determine whether the uncleavable, cytotoxic cell surface form of TNF was cleaved when it interacted with its target, TNF  $\Delta 1-12$  mutant-transfected cells were cocultivated for various times with either TNF-sensitive L929 cells or TNF-insensitive CHO cells. Post incubation, mixed cell populations were lysed and subjected to Western analysis. Western blots were probed with anti-TNF antiserum. Under no conditions could we detect any processing of the uncleavable 26 kd molecule. Reconstruction experiments in the presence and absence of coinubation lysates indicate that our detection sensitivity in these experiments was  $<7$  pg of TNF per sample (Figure 5 and data not shown).

#### 26 kd Transmembrane TNF Is Not Synthesized to Protect Producer Cells from TNF Toxicity during Translation and Release

The 26 kd transmembrane TNF molecule might also function as a prohormone form of the molecule that serves to protect the TNF-producing cell from the potential ravages of intercellular 17 kd TNF. To test this hypothesis, we generated a mutant TNF gene in which we had substituted the DNA sequence encoding the human  $\gamma$ -interferon signal peptide for the codons encoding the 76 amino acid transmembrane leader of 26 kd TNF (Figure 6). Upon transfection into a cell, such a gene should encode a TNF molecule of  $\sim 17$  kd that is cotranslationally inserted into the lumen of the rough endoplasmic reticulum and subsequently secreted. If 17 kd TNF is toxic to the producer cell in either the rough endoplasmic reticulum or the secretory apparatus, then stable transfectants producing 17 kd TNF should be unobtainable. However, after cotransfection and selection with a neo<sup>r</sup> plasmid, 17 kd TNF producers can be obtained (Figure 4, panel 3B). Metabolically labeled cell lysates and cell supernatants derived from cell lines producing this mutant, TNF  $\gamma$  SIG, are found to contain only the 17 kd immunoreactive form of TNF (Figure 3, lanes K and L). The TNF molecules found in the supernatants of such producer cells are biologically active as well. When compared with their wild-type counterparts, TNF  $\gamma$  SIG cell lines produce  $\sim 5$ -fold more secretable TNF per unit time (data not shown).

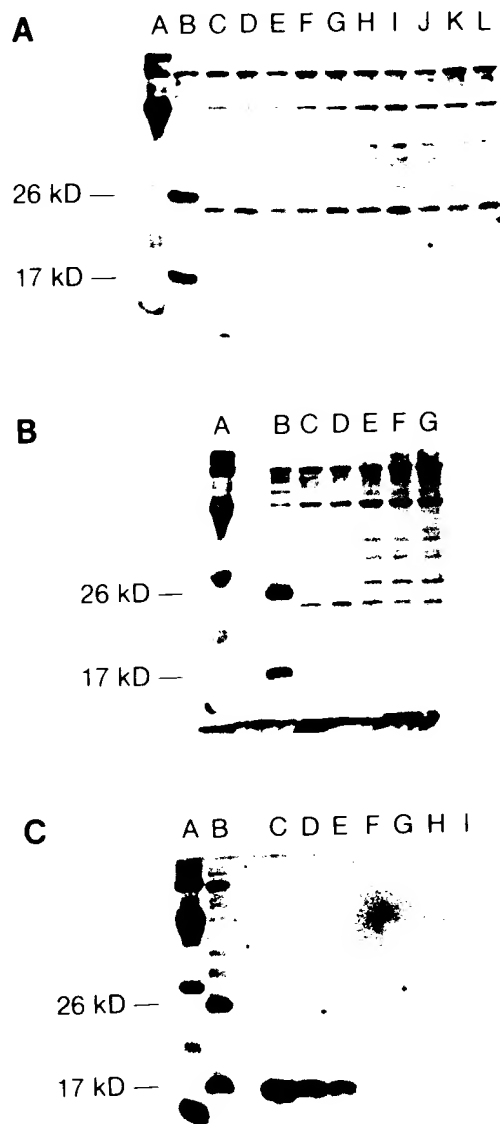


Figure 5. Cleavage Analysis of the Uncleavable Mutant  $\Delta 1-12$  during Killing of TNF-Sensitive Target Cells

Cells producing the TNF mutant  $\Delta 1-12$  were coincubated with media alone, TNF-sensitive L929 cells, or TNF-resistant CHO cells for 0.5, 6, 12, 18, and 24 hr, after which lysates of the cocultivations were subjected to Western analysis.

(A) Lane A, molecular weight markers (14, 21, 30, 46, 69, 92, and 200 kd); lane B, 26 kd and 17 kd TNF markers; lanes C–G,  $\Delta 1-12$  mutant + media at 0.5, 6, 12, 18, and 24 hr; lanes H–L,  $\Delta 1-12$  mutant + L929 cells at 0.5, 6, 12, 18, and 24 hr.

(B) Lane A, molecular weight markers; lane B, 26 kd and 17 kd TNF markers; lanes C–G,  $\Delta 1-12$  + CHO cells at 0.5, 6, 12, 18, and 24 hr. (C) Sensitivity titration of Western analysis for 17 kd TNF. Lane A, molecular weight markers; lane B, 26 kd and 17 kd TNF markers; lanes C–I, decreasing amounts of recombinant TNF: 1.95 ng, 488.5 pg, 122 pg, 30.55 pg, 7.65 pg, 1.9 pg, 478 fg, 119.5 fg, 29.9 fg, and 7.5 fg.

#### Discussion

The experiments described in this paper were conducted to test the hypothesis that the complex physiology of TNF could be a reflection of differences in which the TNF mole-

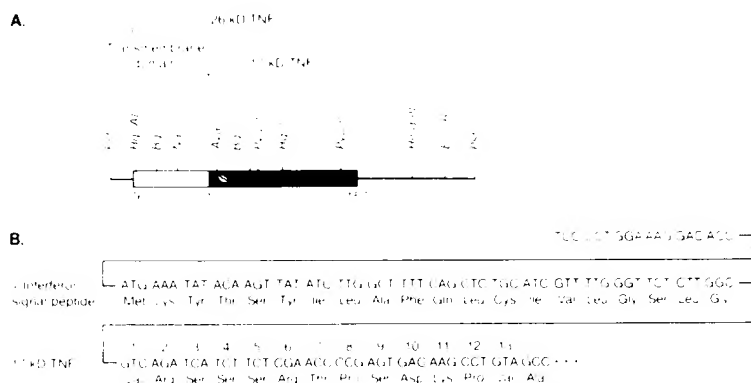


Figure 6. Schematic Depiction of TNF  $\gamma$  SIG Mutant

(A) Diagram of wild-type TNF cDNA.  
(B) Nucleotide and amino acid sequence of  $\gamma$ -interferon signal peptide replacing the transmembrane domain of wild-type TNF in the TNF  $\gamma$  SIG mutant.

cule is presented as a transmembrane molecule or as a secreted molecule. Our first step was to generate, through genetic engineering, a pure population of either the transmembrane form of the molecule or the secretory component. During our attempts to generate an uncleavable form of 26 kd TNF through site-directed mutagenesis, we found that, at least in the NIH 3T3 and CHO cells tested, there existed a second cleavage site in the 26 kd TNF precursor. This second site appears to be flanked by codons 12 and 13 of the gene sequence encoding the 17 kd, mature form of TNF. Our findings are different than those reported by Cseh and Beutler (1989). For murine TNF, they report a scission in the transmembrane domain of the molecule ten amino acids before the natural cleavage site. This difference is possibly due to the different species analyzed, the different cell lines analyzed, and the different methods employed.

In experiments with the uncleavable mutant  $\Delta 1-12$ , we were able to determine that this mutant 26 kd form of the molecule, when it decorates the cell surface, can kill both tumor targets and virally infected cells by cell-to-cell contact. Our plaque assay for tumor cell killing demonstrates that the killing is not due to the production of any diffusible substance. This observation is not without precedent. The elegant studies of Wong et al. (1989), Brachman et al. (1989), and Anklesaria et al. (1990) demonstrate the bioactivity of mutant uncleavable cell surface transmembrane forms of transforming growth factor  $\alpha$ .

Our results strongly suggest that the TNF need not enter a target cell to kill it. Western analysis of whole-cell lysates of TNF-susceptible cells coincubated with cells producing the TNF mutant  $\Delta 1-12$  reveals that, within the limits of our detection technology, no processing of the 26 kd form to the 17 kd form occurs. We cannot of course rule out the possibility that there are a few undetectable, cytotoxic molecules that are cleaved from mutant TNF 26 kd form and subsequently enter the target cell and kill. If this were the case, one would expect to see some diffusion of cytotoxicity in the plaque assay, as least from the largest colonies producing the mutant, uncleavable TNFs, because such colonies produce the most TNF, and diffusion through soft agar is concentration dependent. No diffu-

sion zone, large or small, is detected around even the largest TNF  $\Delta 1-12$  producer colonies. One possibility that we cannot rule out is that, upon binding to a receptor on the target cell, an undetectable amount of the  $\Delta 1-12$  TNF mutant 26 kd molecule is cleaved and internalized, and subsequently kills the target. Such a molecule would not diffuse and thus would not be detected in the plaque assay. The production of pure preparations of the TNF receptor (Loetscher et al., 1990; Schall et al., 1990) expressed from molecular clones should enable us to answer this question.

The TNF mutant  $\Delta 1+12$ , when presented on the surface of a producer cell, kills target cells. Culture supernatants derived from these cells manifest no cytotoxicity in any of our bioassays. Therefore, we were surprised that the extracellular cleavage of this molecule results in the appearance of an immunoreactive 17 kd TNF molecule in the culture supernatants. The essential difference between this mutant and TNF  $\Delta 1-12$  is that the amino acids +2 through +11 remain intact. It appears that the retention of these amino acids permits the cellular surface molecule to be cleaved extracellularly and that this extracellular cleavage inactivates the 17 kd molecule.

When presented on the surface of a producer cell, the TNF mutant  $\Delta 1+13$  does not kill target cells. Culture supernatants derived from these cells also manifest no cytotoxicity in any of our bioassays. This cell surface 26 kd molecule is also cleaved extracellularly. The essential difference between this mutant and TNF  $\Delta 1+12$  is that amino acid 13 is deleted instead of amino acid 12. How these subtle amino acid differences affect the bioactivity of TNF remains a mystery, but an analysis of this problem may provide insight into the mechanism of action of TNF.

The results of these experiments have enabled us to generate: first, transplantable cell lines producing wild-type TNF, a cytotoxic uncleavable 26 kd TNF mutant, and a solely secreted form of 17 kd TNF; and second, infectious recombinant retroviruses encoding these forms of TNF. Such reagents should enable us to separate the pathologies associated with these different forms of TNF in animals in model systems such as these described by Oliff et al. (1987). These experiments are underway.

## Experimental Procedures

### Cell Culture

L929 and LM cells were obtained from the American Type Culture Collection (Rockville, MD) and were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS, GIBCO). NIH 3T3 cells were also obtained from ATCC and were grown in DMEM with 10% calf serum (GIBCO). Mouse C3HA fibroblasts were derived in DMEM, 10% FCS as described by Gooding (1979). Human KB cell maintenance and adenovirus preparation were as described by Green and Wold (1979). CHO cells were maintained in DMEM, 10% FCS and were a gift from Allen Oliff.

d762 is derived from the Ad2-Ad5 recombinant virus rec700 by deletion of the region encoding the E3-14.7 kD protein, leaving the remainder of the E3 region intact (W. S. M. Wold, personal communication).

### Plasmid Constructions

pRV is a pUC derivative of pFVXM, which was described previously (Kriegler et al., 1988). The PstI fragment containing the human TNF cDNA gene was excised from B11 (Wang et al., 1985) and inserted into the PstI site of pRV to produce pRVTNF.

TNF mutants were constructed by established techniques of site-directed mutagenesis (Kramer et al., 1984). The PstI fragment of each TNF mutant was cloned into the PstI site of pRV. pRVTNFΔ(1-12) was generated by using oligonucleotide 5'-TACAACATGGGCTACTGCGCTGGCCAGAGG-3', which deleted the DNA sequence that codes for amino acids +1 through +12. pRVTNFΔ(1+12) was generated by using oligonucleotides 5'-TCGAGAAGATGATCTTGCCTGGGCCAGAGG-3', which deleted amino acid +1, and 5'-TACAACATGGGCTACTTGTCACTCGGGGT-3', which deleted amino acid +12. pRVTNFΔ(1+13) was generated by using the oligonucleotide that deleted amino acid +1 and oligonucleotide 5'-TGCTACAACATGGGCCAGGCTTGTCACCTGG-3', which deleted amino acid +13. pRVTNF<sub>Y</sub>SIG was generated by using oligonucleotide 5'-TCGAGAAGATGATCTGACGCCAAGAGAACCCAAAACGATGCAGAGCTGAAAAGCCCAAGATATACTTGATATTTT-CATGGTGTCTCTCCAGGGG-3', which exchanged the DNA sequence that codes for amino acids -76 through -1 of TNF (the leader peptide) for the DNA sequence that codes for amino acids -20 through -1 of γ-interferon (the signal peptide, Gray et al., 1982).

β-actin-neo was constructed from the 4.3 kb EcoRI-AluI fragment from the human β-actin gene isolate p14Tβ-17 (Leavitt et al., 1984), the BglII fragment containing the neomycin resistance gene from the TN5 transposon (Beck et al., 1982), and the BamHI-ClaI fragment from pcDV1 (Okayama and Berg, 1983), which contains the pBR322 ampicillin resistance gene, bacterial origin of replication, and the SV40 late region polyadenylation signal.

Plasmid DNA was prepared according to the procedure of Birnboim and Doly (1979), banded twice in cesium chloride, and exhaustively dialyzed in TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA).

### DNA Transfection

NIH 3T3 cells were seeded at  $5 \times 10^5$  cells/100 mm dish and cotransfected with 1 μg of β-actin-neo and 10 μg of either pRV or the pRVTNF series of plasmids using conditions as described previously (Kriegler et al., 1984).

### L929 Plaque Assay

Culture dishes with G418-resistant colonies were overlaid with L929 cells at a density of  $7.3 \times 10^4$  cells/cm<sup>2</sup>. When the cells attached, after 30 min, the medium was aspirated and the cells were overlaid with DMEM supplemented with 10% FCS and 0.9% Noble agar. After incubation at 37°C for 48 hr, the agar was carefully removed from plates and cells were fixed and stained in a solution of PBS, 1.2% glutaraldehyde, 0.1% methylene blue.

### Cytotoxicity Assays

On day 1, C3HA cells were infected with Ad5 or d762 at 60 pfu/cell (>95% infection) or mock infected 3 hr after plating at  $1 \times 10^6$  cells/100 mm tissue culture dish. Two hours after infection, cells were washed and 200 μCi of Na<sub>2</sub><sup>51</sup>CrO<sub>4</sub> (New England Nuclear) was added to each dish and incubated overnight. On day 2, effector cells were

trypsinized, pelleted, and resuspended at  $2 \times 10^6$  cells/ml. Cells were plated into 96-well flat-bottomed plates at varying densities. Two hours later supernatants were removed and 100 μl of fresh medium was added. <sup>51</sup>Cr-labeled target cells were trypsinized, pelleted, and resuspended to  $1 \times 10^5$  cells/ml. Target cells (100 μl) were added to each well containing the effector cells and incubated for 20 hr at 37°C and 8% CO<sub>2</sub>. Plates were centrifuged at  $180 \times g$  for 10 min, and a 100 μl sample of supernatant was removed and counted on a Cobra B5005 gamma counter. Results are expressed as the percent of specific <sup>51</sup>Cr release, which equals 100 times <sup>51</sup>Cr cpm released from cells incubated with effector cells minus <sup>51</sup>Cr cpm released in the presence of medium alone divided by <sup>51</sup>Cr cpm released in 1 N HCl (maximum release) minus <sup>51</sup>Cr cpm released in medium alone. Spontaneous release of <sup>51</sup>Cr by cells incubated in medium alone ranged from 29% to 48%.

LM cells were not infected with viruses but were <sup>51</sup>Cr labeled and incubated with effector cells and processed as above.

### Metabolic Labeling of Cells

Cell populations were labeled with [<sup>35</sup>S]cysteine as described previously (Kriegler, 1990).

### Radioiodination

Intact cells were radioiodinated in 24-well plates with <sup>125</sup>I and enzymebeads (Bio-Rad) according to the manufacturer's instructions.

### Immunoprecipitation

<sup>35</sup>S- or <sup>125</sup>I-labeled samples were immunoprecipitated with rabbit anti-human rTNF (Cetus) antisera following conditions described previously (Kriegler, 1990).

### Western Analysis

Samples were resolved on 12% SDS-PAGE and electrophoretically transferred to nitrocellulose membranes (Towbin et al., 1979). Filters were blocked and reacted with antibody. The bound antibody was detected nonisotopically by reacting with goat anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad) followed by incubation with either peroxide and 3,3',5,5'-tetramethylbenzidine (Wong et al., 1986) or enhanced chemiluminescence reagents (ECL; Amersham), following manufacturer's instructions and exposing XAR-5 X-ray film (Kodak) or Hyperfilm (Amersham) to the ECL-treated filter.

### Cocultivation and Cleavage Analysis

TNF Δ(1-12) cells were seeded at  $4 \times 10^6$  cells/60 mm dish. Twenty-four hours later these cultures were overlaid with either no cells,  $4 \times 10^6$  L929 cells, or  $4 \times 10^6$  CHO cells. Cultures were lysed at 0.5, 6.0, 12.0, 18.0, and 24.0 hr after overlay. Cell lysates were subjected to Western analysis.

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# A Novel Form of TNF/Cachectin Is a Cell Surface Cytotoxic Transmembrane Protein: Ramifications for the Complex Physiology of TNF

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## Summary

**Tumor necrosis factor (TNF) is a monocyte-derived cytotoxin that has been implicated in tumor regression, septic shock, and cachexia. The mechanism by which TNF induces these different disease states is unclear. We have identified and characterized a novel, rapidly inducible cell surface cytotoxic integral transmembrane form of TNF. The existence and behavior of this novel form of TNF may explain the complex physiology of this molecule. We suggest that activated monocytes synthesize transmembrane TNF at the site of inflammation and kill their targets by either cell-to-cell contact or local release of the TNF secretory component. In contrast, septic shock and cachexia may result from either acute or chronic systemic activation of monocytes, resulting in the widespread release of TNF secretory component into the circulation of the affected individual. We further suggest that cell borne cytokines and cytotoxins may be the primary mediators of directed inflammatory responses.**

## Introduction

Tumor necrosis factor is the name given to a factor that appears in the serum of affected animals during the acute phase of an inflammatory response. This factor is cytotoxic for some tumor cell lines in vitro and causes the necrosis of certain tumors in vivo (for review, see Old, 1985). The phenomenon was first described late in the last century when physicians noted rare spontaneous regressions of tumors in cancer patients. In several instances the regression occurred in coincidence with the onset of an infectious disease.

In an attempt to recreate this situation, physicians infected terminally ill cancer patients with a variety of infectious agents. Unfortunately, they were unable to control the severity of the infections and, as a result, did more harm than good. To overcome this problem, patients were injected with lysed filtrates of selected bacteria. This approach proved more successful than the former. In fact, one particular lysate preparation, Coley's Toxin, was, until the advent of chemotherapy and radiation therapy in 1934, the only method approved for the systemic treatment of cancer.

After 1934, clinical research on bacterial toxins as antitumor agents subsided. However, research on the effects of bacterial toxins on murine tumors continued. Further research indicated that the bacterial component essential for cell killing was the lipopolysaccharide (LPS) component of the bacterial cell wall. It appeared that, in the correct setting, the administration of LPS to mice could induce an activity in mouse serum that was cytotoxic to some tumor cell lines in vitro. This in vitro activity was called tumor necrosis factor. The availability of such in vitro assays for TNF led to the isolation, purification, and subsequent molecular cloning of the gene encoding human tumor necrosis factor.

Tumor necrosis factor exhibits multiple biological activities (Old, 1985). It has also been shown to be a critical factor involved in the onset of septic shock. In addition, TNF is identical to cachectin, a serum borne factor associated with cachexia, an emaciated condition of the body associated with chronic illness (Beutler et al., 1985a, 1985b; Beutler and Cerami, 1986; Tracey et al., 1986, 1987; Old, 1987; Oliff et al., 1987). This factor has been reported to have antiviral activity as well (Wong and Goeddel, 1986).

Human tumor necrosis factor has been identified as a 17 kd polypeptide that is secreted by activated monocytes. It is generally assumed that the synthesis and secretion of TNF begins with the elaboration of an unusually long 76 amino acid signal peptide into the rough endoplasmic reticulum, and that, after cleavage of the signal peptide, the elongation of a 17 kd peptide ensues (Pennica et al., 1984; Wang et al., 1985; Shirai et al., 1985). Presumably, this molecule is then secreted via normal secretory pathways. The possibility that the 76 amino acid signal peptide might serve another function has been suggested by Muller and colleagues (1986). They reported that TNF, when translated in vitro in the presence of microsomes, was not processed. However, no such unprocessed counterpart has been reported in vivo. Our studies were undertaken in an attempt to understand the relationship between the induction of tumor cell killing, septic shock, and cachexia by TNF. We postulated that different forms of the TNF molecule might induce different physiologic responses. Therefore, we began a search for other forms of tumor necrosis factor.

## Results

### Human Monocytes Synthesize a High Molecular Weight Form of Tumor Necrosis Factor

Human monocytes can be induced to synthesize TNF by exposure to both LPS and PMA. To examine the TNF produced in human monocytes, we treated monocytes with LPS and PMA after which both untreated and treated (induced) monocytes were lysed and subjected to Western analysis employing a polyclonal antibody to human tumor necrosis factor as the antibody probe. The results of that analysis are shown in Figure 1.

The TNF polyclonal antibody detects a 26 kd protein in

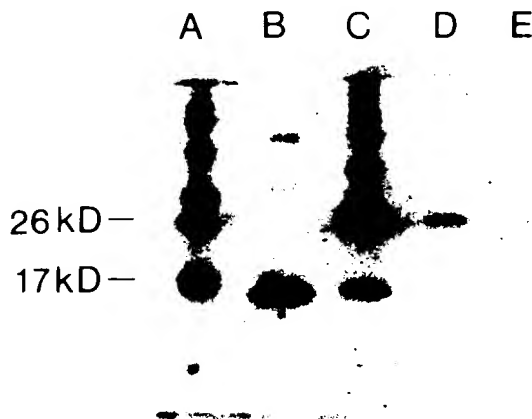


Figure 1. Western Analysis of Uninduced and Induced Human Monocytes

Human monocytes were purified from peripheral blood by centrifugation. After centrifugation the monocyte fraction was divided in half, and one portion was exposed to PMA + LPS and allowed to incubate for 1 hr after which the cells were lysed subjected to electrophoresis in a 12% polyacrylamide gel followed by Western analysis. Anti-TNF antibody was used to probe the blot after which the blot was incubated with radiolabeled protein A. This figure is an autoradiogram of that Western blot. Lane A, molecular weight markers; lane B, E. coli produced 17 kD TNF; lane C, TNF 6.8 cell lysate; lane D, induced human monocytes; lane E, uninduced human monocytes. TNF 6.8 is a cell line isolated from a population of  $\psi$ am cells transfected with pFVXMTNF6 plasmid containing a retroviral vector into which the human TNF cDNA clone B11 had been inserted.

induced human monocytes that is not detected in uninduced human monocytes. This same polyclonal antibody detects recombinant, 17 kD, TNF as well. When purified monocytes induced in a similar manner were metabolically labeled with  $^{35}$ S-cysteine and subjected to immunoprecipitation analysis with anti-TNF antisera, a 26 kD protein was precipitated and could be detected on the autoradiogram of the gel.

We were surprised when we detected the 26 kD peptide for several reasons. First of all, the 26 kD protein is the size of the protein predicted by the TNF cDNA is the leader sequence, the putative signal sequence, is not removed. Second, if this 26 kD protein is in fact a 17 kD TNF precursor and if the 76 amino acid leader is the TNF secretory signal, then the mere fact that the precursor is detectable is remarkable. This is because during elaboration of secretory proteins into the endoplasmic reticulum, amino-terminal signal peptides are cleaved cotranslationally (Walter and Lingappa, 1986).

#### Tissue Culture Cells Transfected with the TNF cDNA Synthesize Both 26 kD and 17 kD TNF

In an attempt to generate cell lines that constitutively produce large amounts of TNF, we subcloned the TNF cDNA into a retroviral vector, pFVXM (see Experimental Procedures). This construction was cotransfected with the selectable marker pEVX neo into NIH 3T3 cells, and the

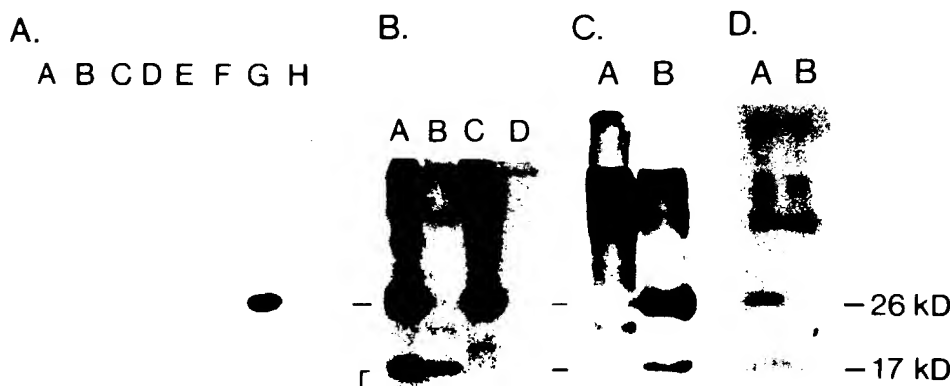


Figure 2. Immunoprecipitation and Competition Analysis of 17 kD and 26 kD TNF

Cells were labeled with  $^{35}$ S-cysteine or  $^{35}$ S-methionine, subjected to immunoprecipitation, electrophoresis in a 12% polyacrylamide gel, and autoradiography. Autoradiograms of these gels are shown.

(A): Immunoprecipitation analysis of  $^{35}$ S-cysteine labeled  $\psi$ am and TNF 6.8 cell lines with preimmune and anti-17 kD TNF antisera. Lane A:  $\psi$ am cell lysate precipitated with preimmune serum; lane B:  $\psi$ am cell supernatant precipitated with preimmune serum; lane C:  $\psi$ am cell lysate precipitated with anti-TNF antiserum; lane D:  $\psi$ am cell supernatant precipitated with anti-17 kD TNF antiserum; lane E: TNF 6.8 cell lysate precipitated with preimmune serum; lane F: TNF 6.8 cell supernatant precipitated with preimmune serum; lane G: TNF 6.8 cell lysate precipitated with anti-17 kD TNF antiserum; lane H: TNF 6.8 cell supernatant precipitated with anti-17 kD TNF antiserum.

(B): Immunoprecipitation with anti-17 kD TNF antiserum of 17 kD and 26 kD TNF labeled with  $^{35}$ S-cysteine and  $^{35}$ S-methionine from TNF 6.8 cells. Lane A:  $^{35}$ S-cysteine labeled cell lysate; lane B:  $^{35}$ S-cysteine labeled cell supernatant; lane C:  $^{35}$ S-methionine labeled cell lysate; lane D:  $^{35}$ S-methionine labeled cell supernatant.

(C): Competition binding analysis of  $^{35}$ S-cysteine labeled TNF 6.8 cell lysates. Lane A:  $^{35}$ S-cysteine labeled cell lysate and unlabeled recombinant TNF immunoprecipitated with anti-17 kD TNF antiserum; lane B:  $^{35}$ S-cysteine labeled cell lysate precipitated with anti-17 kD TNF antiserum.

(D): Competition binding analysis of  $^{35}$ S-cysteine labeled cell lysates of induced human monocytes. Lane A:  $^{35}$ S-cysteine labeled induced human monocyte cell lysate immunoprecipitated with anti-17 kD TNF antiserum; lane B:  $^{35}$ S-cysteine labeled induced human monocyte cell lysate and unlabeled (50  $\mu$ g) recombinant TNF precipitated with anti-17 kD TNF antiserum.

cotransfected cells were selected for resistance to G418. Isolated transfectants were analyzed for the production of TNF by plaque assay, cytotoxicity, and immunoprecipitation analysis.

The results of the cytotoxic TNF assay revealed that the transfected clones secreted as high as  $1.5 \times 10^3$  U/ml/6 hr of TNF. Immunoprecipitation analysis of metabolically labeled cell lysates and cell supernatants (media) revealed the presence of two proteins in the cell lysates, one at 17 kd, migrating with recombinant TNF and one, the major band, at 26 kd, migrating with the 26 kd molecule detected in induced monocytes. Immunoprecipitation analysis of supernatants derived from metabolically labeled cells revealed the presence of a single protein migrating at 17 kd, migrating with recombinant TNF as well as the 17 kd band present in the cell lysates (Figures 2A and 2B). Neither the 26 kd molecule nor the 17 kd molecule could be detected with preimmune serum (Figure 2A). The putative 26 kd TNF molecule could be labeled with both  $^{35}$ S-methionine and  $^{35}$ S-cysteine. However, the 17 kd molecule could only be labeled with  $^{35}$ S-cysteine, not  $^{35}$ S-methionine (Figure 2B). Thus, if the 26 kd molecule was the precursor for the 17 kd molecule, some processing of the 26 kd molecule, resulting in the removal of the 2 methionine residues encoded by the TNF cDNA, must have occurred at the amino terminus.

To verify the identity of 26 kd and 17 kd molecules detected in these experiments, we conducted an antibody competition experiment. Both the labeled 26 kd and 17 kd species could be displaced with recombinant TNF, thus indicating that the 26 kd protein and the 17 kd protein detected in our analyses share epitopes with recombinant TNF (Figure 2C). Similar results were obtained with 26 kd and 17 kd TNF derived from radiolabeled monocytes (Figure 2D).

#### The 26 kd Protein Is the Precursor for the 17 kd TNF Molecule

To determine if the 26 kd protein is the precursor for the 17 kd TNF, we conducted a pulse-chase experiment (Figure 3). Examination of the autoradiogram revealed the presence of the 26 kd protein in the cell lysates where no 17 kd TNF was detected at the zero timepoint. Fifteen minutes after labeling, both the 26 kd protein and 17 kd TNF were detected. The remaining timepoints indicated that as the amount of the 26 kd molecule decreases, the amount of the 17 kd molecule increases.

Immunoprecipitation analysis of supernatants derived from the identical pulse-labeled cells were analyzed (Figure 3). As the 26 kd protein decreases in the cell lysates, 17 kd TNF increases in the cell supernatants. If we account for the fact that the 26 kd protein contains four cysteines and the 17 kd TNF contains two cysteines, densitometric analysis of the bands demonstrates that virtually all of the 26 kd protein is converted to 17 kd TNF in cell lysates and cell supernatants (data not shown).

If the 26 kd protein is the full-length molecule encoded by the TNF cDNA, then the 26 kd molecule we detect in both immunoprecipitation analysis and Western analysis should contain the leader sequence predicted by the se-

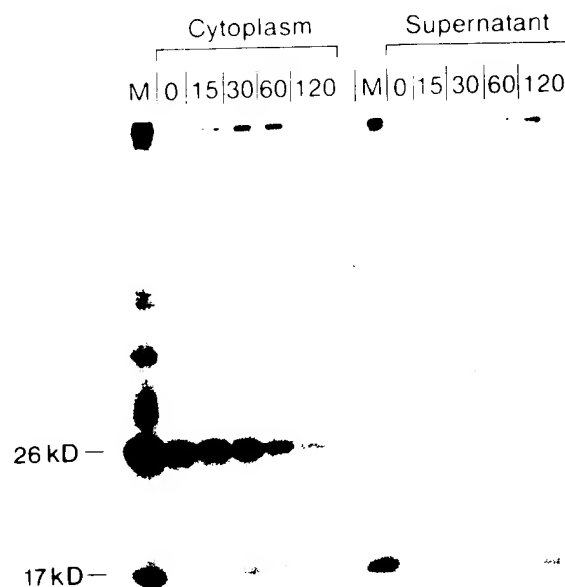


Figure 3. Pulse-Chase Analysis of 26 kd and 17 kd TNF. TNF producing NIH 3T3 cells (line TNF 6.8) were labeled for 15 min with  $^{35}$ S-cysteine and subsequently incubated with excess unlabeled cysteine for the times indicated above each lane. After chase, cell lysates and supernatants were subjected to immunoprecipitation with anti-17 kd TNF antiserum. Immune precipitates were subjected to electrophoresis in a 12% polyacrylamide gel after which the gel was subjected to autoradiography. An autoradiogram of that gel is shown above.

quence encoded by the TNF cDNA. To test this hypothesis, we inoculated four synthetic peptide-KLH conjugates into rabbits, in an attempt to raise antibodies specific for the putative TNF leader sequence. One of the four peptides, TNF 2 (Lys-19 to Cys-30 of the putative signal sequence), raised antisera that detect the 26 kd protein in Western analysis (Figure 4A), but not the 17 kd molecule.

#### 26 kd TNF Is a Membrane-Associated Protein

The studies described above indicate that the 76 amino acid leader is not a classical secretory protein signal peptide. Examination of the primary sequence of 26 kd TNF leader reveals a hydrophobic domain of approximately 20 amino acids flanked by two hydrophilic domains characteristic of an integral transmembrane protein. This observation led us to test the notion that 26 kd TNF is a membrane-associated protein. To accomplish this task, a TNF producing cell line was sonicated and, after multiple steps of differential centrifugation, total cellular membranes were fractionated from the cytosol. Both cytosol and membrane fractions were tested by Western analysis for the presence of 26 kd TNF. In fractionated cells, the vast majority of the 26 kd TNF in the cell is membrane-associated (Figure 4B).

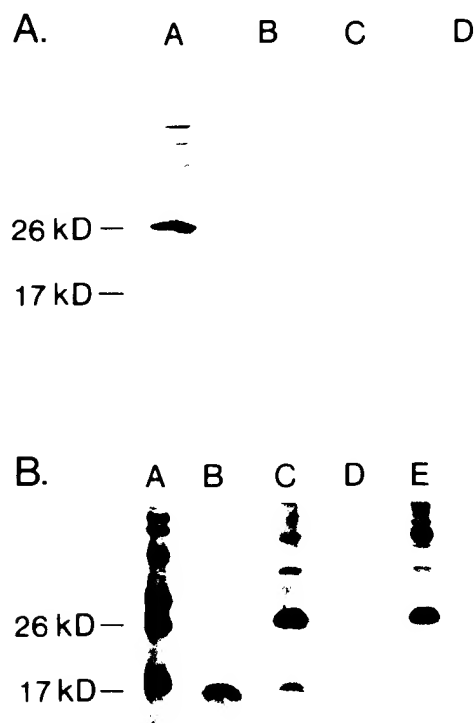


Figure 4. Western Analysis of the Molecular Structure and Membrane Association of 17 kd and 26 kd TNF

Cell lysates, supernatants, and membrane fractions were prepared as described in Experimental Procedures. Samples were subjected to electrophoresis in a 12% polyacrylamide gel followed by nonisotopic Western analysis as described by Wong et al. (1986).

(A): Western analysis of cell lysates and supernatants probed with anti-17 kd TNF antiserum and TNF 2 anti-TNF leader antiserum. Lanes A and C: TNF 6.8 cell lysates; lanes B and D: TNF 6.8 cell supernatants; lanes A and B: anti-17 kd TNF antiserum; lanes C and D: TNF 2 anti-TNF leader antiserum.

(B): Western analysis of cytosol and membrane fractions for 26 kd and 17 kd TNF probed with anti-17 kd TNF antiserum. Lane A: molecular weight markers; lane B: recombinant TNF; lane C: TNF 6.8 cell lysate; lane D: cytosol fraction of fractionated TNF 6.8 cells; lane E: membrane fraction of fractionated TNF 6.8 cells.

### 26 kd TNF Is an Integral Transmembrane Protein

To further study the nature of the association of 26 kd TNF with membranes, we produced pure populations of 26 kd TNF by *in vitro* translation. We generated two recombinant DNA molecules to serve as DNA templates by subcloning two TNF cDNAs into a bacterial plasmid that contains a T7 promoter. One inserted TNF cDNA, the wild-type cDNA, contains the codon encoding the natural N-terminal methionine at the 5' end of the TNF structural gene. The other inserted TNF cDNA was engineered, via site-directed mutagenesis, such that the N-terminal methionine of 26 kd TNF, along with the remaining 5' untranslated region of the cDNA, abuts the codon that encodes the N-terminal valine that is exposed after processing of 26 kd TNF. These plasmids were transcribed *in vitro* and used to program rabbit reticulocyte lysates in the presence of dog pancreatic microsomes. The transcript encoding 26 kd

TNF only programs the synthesis of a 26 kd immunoprecipitable protein. The transcript encoding 17 kd TNF only programs the synthesis of a 17 kd protein. When tested for cytotoxic activity in the L929 cell killing assay (Wang et al., 1985), 26 kd TNF was found to have no effect (data not shown). However, an equal amount of *in vitro* synthesized 17 kd TNF was shown to contain over 1000 units per milliliter of activity in the same cytotoxic cell assay (data not shown).

Comparison of the *in vitro* translation products encoded by the 26 kd TNF transcripts in the presence of dog pancreatic microsomes reveals that microsomes do not alter the molecular weight of the 26 kd TNF *in vitro* translation product. The 26 kd molecule was not processed to 17 kd (Figure 5B). This observation supports the contention that the 76 amino acid leader is not a signal peptide but rather serves another function. The observations that the putative "signal peptide" was not cleaved by the microsomes and that 26 kd TNF is membrane-associated led us to test the hypothesis that the 76 amino acid leader sequence might function as a transmembrane domain. The next two experiments were designed to determine the nature of the 26 kd TNF interaction with the membrane first observed in the experiment described in Figure 4.

To rule out the possibility that 26 kd TNF was included in the membrane fraction due to preferential encapsulation into microsomes during the process of cell disruption, 26 kd TNF was synthesized *in vitro* in the presence of dog pancreatic microsomes. The translation reaction was divided and half of the reaction was incubated in 1× PBS while the other half of the reaction was incubated at pH 11.5, a condition which is known to open microsomes without disrupting their membranes. Under such conditions, it has been shown that proteins encapsulated by microsomes are released; however, proteins tightly associated with membranes are retained in the membrane fraction after centrifugation (Teixido et al., 1987). The results of immunoprecipitation analysis of these reactions are shown in Figure 5A. Alkali treatment of the 26 kd TNF translated with microsomes does not dissociate the 26 kd molecule from the membrane (Figure 5A, lanes E and F). This association can be disrupted by addition of detergent to the pellet (data not shown).

If 26 kd TNF is a transmembrane protein, then a portion of the protein should remain exposed after the protein is incorporated into a microsome. If the 26 kd TNF/microsome complexes are subsequently exposed to a protease, we expect the exposed portion of 26 kd TNF to be digested and a portion of the 26 kd molecule to be protected from digestion. The results of such a digestion/immunoprecipitation experiment are shown in Figure 5B. This experiment reveals that a portion of 26 kd TNF is susceptible to proteolytic digestion (Figure 5B, lanes H and I), microsome-associated, protease-treated 26 kd TNF migrates at a slightly lower molecular weight, approximately 24 kd.

In an attempt to determine the orientation of the 26 kd molecule in the microsomal membrane, we took advantage of the fact that the only methionine residues in the 26 kd TNF molecule are at the amino terminus of the molecule at positions 1 and 6. If the amino terminus of the 26



Figure 5. Analysis of In Vitro Synthesized 26 kd TNF and Its Association with the Microsomal Membrane

T7 transcripts encoding 26 kd TNF were translated in vitro treated as described below and in Experimental Procedures, immunoprecipitated, and subjected to electrophoresis in a 12% polyacrylamide gel. The resultant gels were subjected to autoradiography and autoradiography. Autoradiograms of those gels are shown below.

(A) Alkali treatment and ultracentrifugation analysis of  $^{35}\text{S}$ -cysteine labeled 26 kd TNF translated in vitro in the presence of microsomes. All samples were immunoprecipitated with anti 17 kd TNF antiserum. Lane A: TNF 6.8 cell lysate; lane B: in vitro translated 26 kd TNF; lane C: neutral supernatants of pelleted microsomal preparations; lane D: pellet of neutral microsomal preparations; lane E: supernatant of pelleted alkaline microsomal preparations; lane F: pellet of alkaline microsomal preparations

(B) Proteinase K digestion of  $^{35}\text{S}$ -cysteine labeled 26 kd TNF synthesized in vitro in the absence and presence of microsomes. Lanes A, B, D, F, and H immunoprecipitated with anti-17 kd TNF antiserum. Lanes C, E, G, and I immunoprecipitated with anti-TNF 2 TNF leader antiserum. Lane A: TNF 6.8 cell lysate; lanes B and C: 26 kd TNF synthesized in the absence of microsomes; lanes D and E: 26 kd TNF synthesized in the absence of microsomes digested with proteinase K; lanes F and G: 26 kd TNF synthesized in the presence of microsomes; lanes H and

kd molecule is outside the microsome, then these two methionine residues should be subject to proteolysis as the 26 kd molecule is converted to the 24 kd counterpart. To test this possibility, in vitro translation of the mRNA encoding 26 kd TNF was carried out in either the presence of  $^{35}\text{S}$ -cysteine or  $^{35}\text{S}$ -methionine, in the presence of microsomes, and subjected to proteolysis.

Digestion of the  $^{35}\text{S}$ -methionine labeled 26 kd TNF in the presence of microsomes results in the complete disappearance of the 26 kd TNF molecule. In the experiment in Figure 5C, the  $^{35}\text{S}$ -cysteine labeled 26 kd TNF molecules can be tracked to the 24 kd digestion product because 26 kd TNF contains four cysteine residues dispersed throughout the molecule. Partial digestion of the molecule from either the carboxyl or amino terminus, removing as much as 2 kd of the 26 kd molecule, cannot remove all of the cysteine residues.

However, removal of six amino acids from the amino terminus of  $^{35}\text{S}$ -methionine labeled 26 kd TNF should result in the radioisotopic disappearance of the 24 kd band we detect in the cysteine labeled sample. The 24 kd molecule exists, unlabeled. Thus, as shown in Figure 5C, it appears that the portion of the 26 kd TNF molecule that is exposed to proteolysis outside of the microsome is the amino terminus.

#### Integral Transmembrane TNF Is Cytotoxic

In previous experiments (described above), we showed that in vitro synthesized 26 kd TNF was not cytotoxic. Yet, in vitro synthesized 17 kd TNF was cytotoxic. One interpretation of that data is that 26 kd TNF cannot fold correctly in the absence of microsomes and, incorrectly folded, is not cytotoxic. Having shown that 26 kd TNF is an integral transmembrane protein, we attempted to determine if 26 kd TNF synthesized in vitro in the presence of microsomes might be cytotoxic.

We synthesized 26 kd TNF in vitro in the absence and presence of microsomes. Each reaction was divided in half and one sample of each was sonicated. A sample of the in vitro translation reaction mixtures, containing microsomes, were sonicated to open up the microsomes and thus expose the TNF cytotoxic domain, normally inside the microsome, to the outside. A sample of the in vitro translation reaction mixtures, not containing microsomes, was sonicated to control for the possibility that sonication might cleave the cytotoxic 17 kd secretory component from the 26 kd molecule. Each sample was analyzed for cytotoxicity. Only the sonicated in vitro translation reaction

I, 26 kd TNF synthesized in the presence of microsomes digested with Proteinase K.

(C): Determination of the polarity of integral transmembrane 26 kd TNF in the microsomal bilayer. 26 kd TNF was translated in the presence of microsomes and labeled with either  $^{35}\text{S}$ -cysteine or  $^{35}\text{S}$ -methionine. Half of each reaction was digested with proteinase K. All reactions were immunoprecipitated with anti-17 kd TNF antibody. Lane A: 26 kd TNF marker; lane B:  $^{35}\text{S}$ -cysteine labeled 26 kd TNF undigested with proteinase K; lane C:  $^{35}\text{S}$ -methionine labeled 26 kd TNF undigested with proteinase K; lane D:  $^{35}\text{S}$ -cysteine labeled 26 kd TNF digested with proteinase K; lane E:  $^{35}\text{S}$ -methionine labeled 26 kd TNF digested with proteinase K



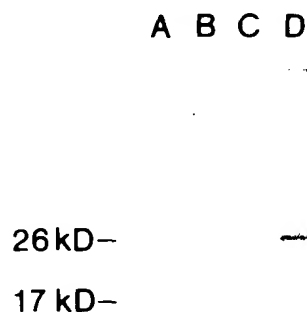


Figure 6. Radioiodination of Cell Surface TNF. Intact cells of a TNF producing cell line (TNF 68) were radioiodinated as described in Experimental Procedures. After radioiodination, cell lysates were immune precipitated with either preimmune or immune antiserum.  
Lane A:  $\mu$ m cells treated with preimmune serum.  
Lane B:  $\mu$ m cells treated with anti-TNF serum.  
Lane C: TNF 68 cells treated with preimmune serum.  
Lane D: TNF 68 cells treated with anti-TNF serum.

mixture containing microsomes displayed cytotoxicity, on L929 cells approximately 50 U/ml in four independent experiments. All of the other samples contained no detectable activity ( $<1.0$  U/ml). Thus we conclude that 26 kD TNF must be inserted into a membrane to exhibit cytotoxicity and that the microsome containing 26 kD TNF must be turned inside-out to display the cytotoxic portion of the molecule.

#### Integral Transmembrane TNF Is Presented by the Cell Membrane

In an attempt to determine if 26 kD TNF is present on the outside of the cell, we radioiodinated intact cells from a TNF producing cell line under conditions in which the viability of the cells was unaffected (see Experimental Procedures). The results of an immunoprecipitation analysis of the radioiodinated cells is shown in Figure 6. Immunoprecipitable peptides at 26 kD and 17 kD were detected in the sample precipitated with anti-TNF antiserum. Therefore, 26 kD integral transmembrane TNF is found on the outside of the cell.

#### Discussion

In addition to tumor regression, tumor necrosis factor is implicated in the induction of a variety of disease states. Recent reports have shown TNF to be an active agent in both cachexia and septic shock (Beutler et al., 1985a; 1985b; Beutler and Cerami, 1986; Tracy et al., 1986; Oliff et al., 1987). How might TNF exhibit such a complex biology?

We embarked on the experiments described in this paper to test the hypothesis that the complex physiology of TNF might be the result of multiple forms of TNF in the body. Upon examination of cell lysates of purified human monocytes induced to synthesize TNF cytotoxic activity, we observed an immunoprecipitable 26 kD protein in the

induced sample that was absent in the uninduced sample. This protein was the size predicted to be encoded by the structural gene if the putative signal peptide was not removed from the 17 kD protein.

Our observation that the 26 kD molecule could be labeled both in vivo and in vitro with  $^{35}$ S-methionine indicated that the first six amino acids were not removed from the 26 kD molecule until it was processed to the 17 kD secretory component. Furthermore, the observation that the 26 kD TNF molecule accumulates in the cell strongly suggested that the biosynthesis and secretion of 17 kD TNF is not typical of secretory proteins in that peptide elongation into the lumen of the endoplasmic reticulum is generally preceded by signal peptide cleavage.

These data suggested to us that the 17 kD component of TNF might be secreted by another mechanism. Perhaps 26 kD TNF was a membrane protein that was clipped to generate the 17 kD secretory component. Examples of such a secretory mechanism have been described previously for the epidermal growth factor (Scott et al., 1983; Gray et al., 1983), TGF $\alpha$  (Bringman et al., 1987; Teixido et al., 1987), and the IgA receptor (Mostov and Blobel, 1982). Our pulse-chase analysis and membrane fractionation studies indicate that this is indeed the case. The 26 kD molecule is the precursor to the 17 kD secretory component. In addition, the 26 kD molecule is an integral transmembrane protein with a rather unique orientation. The cytoplasmic component lies at the amino terminus of the 26 kD molecule, while the cytotoxic, carboxyl terminal portion of the molecule is found either inside the microsome, outside the cell, or both. Radioiodination of intact cells producing TNF indicate that 26 kD TNF decorates the cell surface. Therefore, it appears that our hypothesis is correct; human tumor necrosis factor is a cell surface integral transmembrane protein that must be cleaved to be released into the serum. This observation dramatically alters the way in which we can explain the biology of TNF.

We propose a model for TNF dependent tumor regression in vivo. In our model the two forms of tumor necrosis factor, 26 kD integral transmembrane TNF and the 17 kD secretory component, function in two fundamentally different ways. Initially, monocytes and macrophages are activated and stimulated to migrate to the tumor and/or to the site of inflammation. Such activation leads to the synthesis of 26 kD integral transmembrane TNF. Either this 26 kD molecule is itself cytotoxic, or alternatively, upon degranulation of the macrophage, 26 kD TNF is processed to the 17 kD cytotoxic secretory component. The notion that 26 kD TNF is itself cytotoxic is consistent with the observations of Decker et al. (1987) that paraformaldehyde treated activated macrophages are cytotoxic in a TNF-dependent assay. In either instance the toxicity of TNF will be localized. In the case of 26 kD TNF mediated cytotoxicity, toxicity will be localized because cell to cell contact is required. In the case of 17 kD TNF mediated cytotoxicity, toxicity will be localized because degranulation and subsequent 26 kD to 17 kD TNF processing occurs locally (Figure 7). In either case, systemic TNF toxicity is absent because the toxic molecules are only present in the microenvironment of the activated monocyte or macrophage.

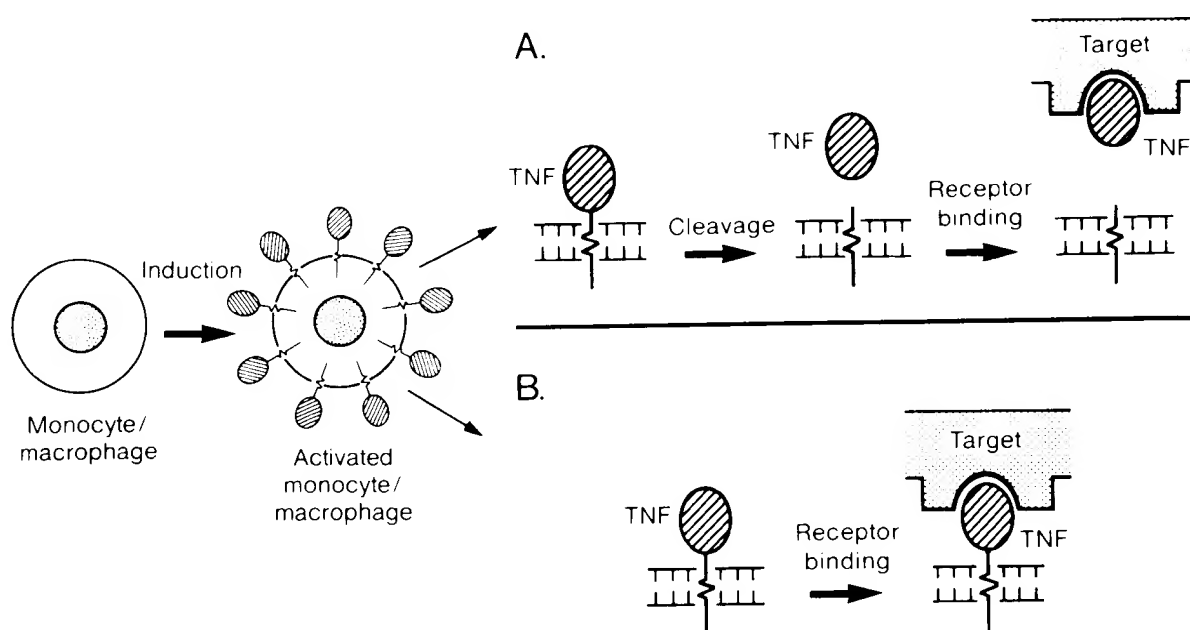


Figure 7. Proposed Mechanism of Action of 26 kd TNF Mediated Cytotoxicity

(A): Cell killing after cleavage and binding of 17 kd secretory component to TNF receptor.

(B): Cell killing by cell to cell contact and subsequent interaction of 26 kd TNF with TNF receptor.

Septic shock and cachexia might appear as a consequence of systemic activation of monocytes and macrophages such that cytotoxic TNF, either 26 kd, 17 kd or both, is no longer localized, resulting in acute systemic toxicity characteristic of septic shock or chronic systemic toxicity characteristic of cachexia. In support of this hypothesis is the observation that similarities exist between the clinical response to exogenously administered 17 kd TNF and elements of the clinical syndrome septic shock. These include hypotension, fever, malaise, gastrointestinal disturbances, and altered mental state (A. Rudolph, personal communication). Experiments designed to test these hypotheses are currently under way.

#### Experimental Procedures

##### Cell Culture

L929 cells were obtained from the American Type Culture Collection (Rockville, MD) and were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (GIBCO).  $\psi$ am cells were obtained from Richard Mulligan (Cone and Mulligan, 1984) and were grown in DMEM supplemented with 10% calf serum (GIBCO).

Human monocytes were purified from human blood by centrifuging first through Ficoll-Paque and Percoll (49.2%) following manufacturer's (Pharmacia) conditions. The enriched population of monocytes and lymphocytes were plated into dishes containing RPMI (GIBCO) supplemented with 20% FCS and incubated for 30 min (37°C). The dishes were extensively rinsed with RPMI, leaving only adherent monocytes. The monocytes were further incubated for 3 h (37°C) in RPMI supplemented with 20% FCS, 100 ng/ml lipopolysaccharide (LPS, Sigma), and 10  $\mu$ g/ml phorbol myristate acetate (PMA, Sigma), to induce tumor necrosis factor synthesis. The L929 cell killing assay was performed as described by Wang et al. (1985).

##### Antisera

Peptides were obtained by solid phase synthesis (Merrifield, 1985). Cysteine was incorporated into the peptides to provide a sulfhydryl

group. The N-maleimido-6-aminocaproyl ester of 1-hydroxy-2-nitro-4-benzenesulfonic acid (mal-sac-HNSA), was used to cross-link the sulfhydryl group of the peptide to either KLH, for immunization, or BSA, for analysis. After an initial inoculation with 500  $\mu$ g of protein or KLH-peptide conjugate in Freund's complete adjuvant, animals were boosted with 250  $\mu$ g of protein or peptide conjugate in Freund's incomplete adjuvant every 3 weeks. Serum samples were tested for immunoreactivity in immunoprecipitation analyses every 3 weeks.

##### Plasmid Constructions

The biology of pEVX has been described (Kriegler et al., 1984). We have shown that the presence of the Moloney murine leukemia virus derived splice donor just 3' to the 5'-LTR dramatically decreases the yield of correctly spliced translational templates of retroviral constructions, carrying multiple exon genes, post transfection, and virus rescue. pEVX was digested partially with SmaI and completely with BglI, and the fragment was removed between these sites (containing the splice donor). An analogous SmaI fragment of the Harvey murine sarcoma virus genome, which we previously determined did not contain a Mo-MuLV splice donor, was substituted. This plasmid was partially digested with BglI and completely with SstII, and the fragment between these two sites was discarded. The ends were made blunt by T4 DNA polymerase and then ligated. The resulting vector, pFVXM, lacks a splice donor and carries a viral packaging signal.

The PstI fragment containing the human TNF cDNA gene was excised from plasmid B11 (Wang et al., 1985) and inserted into the PstI site of pFVXM such that the TNF gene is expressed from the 5' LTR, producing pFVXM-TNF6. The same fragment was cloned into the PstI site of pGem-3 (Promega Biotec) such that the T7 RNA polymerase promoter can synthesize in vitro RNA sense-transcripts, producing pGEM-TNF14.

pGem-TNF $\Delta$ LEAD was constructed by deleting the DNA sequence that codes for amino acids 2 to 76, thereby deleting the leader peptide, by established techniques of site-directed mutagenesis (Kramer et al., 1984). The PstI fragment was inserted into pGem-3 in the same orientation as pGEM-TNF14, producing pGEMTNF $\Delta$ LEAD. pEVX-neo has been described previously (Kriegler et al., 1984).

Plasmid DNA was prepared according to the procedure of Birnboim and Doly (1979), banded twice in cesium chloride, and exhaustively dialyzed in TE Buffer (10 mM Tris [pH 8.0], 1 mM EDTA).

#### DNA Transfection

mam cells were cotransfected with plasmids pFVXM-TNF6 and pEVX-neo as described previously (Kriegler et al., 1984).

#### Plaque Assay of TNF Producing Colonies

Culture dishes with G418-resistant colonies were overlaid with L929 cells at a density of  $7.3 \times 10^4$  cells/cm<sup>2</sup>. When the cells attached, after 30 min, the media was aspirated, and the cells were overlaid with DMEM supplemented with 10% FCS and 0.9% Noble agar. After incubation for 18–24 hr, clones surrounded by a lysed zone of L929 cells were isolated by cloning cylinders and expanded to mass culture. One cloned line, TNF 6.8, which produced the most TNF, was used for further analysis.

#### Metabolic Labeling of Cells

The TNF 6.8 cell line was labeled with <sup>35</sup>S-cysteine as described previously (Kriegler and Botchan, 1983). For timed metabolic labeling experiments, monolayers were pulsed for 15 min with <sup>35</sup>S-cysteine, chased with cold cysteine for the times indicated, and then lysed for analysis.

A population of cells enriched for human monocytes was plated into 601 mm dishes containing RPMI media supplemented with 20% FCS, and incubated for 30 min at 37°C. The dishes were extensively rinsed with RPMI, leaving only adherent monocytes. The monocytes were incubated with cysteine-minus RPMI media supplemented with 100 ng/ml LPS, 10 µg/ml PMA for 30 min at 37°C, after which, 100 µCi of <sup>35</sup>S-cysteine and dialyzed FCS (5% final concentration) was added. Incubation was continued at 37°C for 3 hr, after which the cells were lysed for analysis.

#### Membrane Fractionation

Five 100 mm dishes of subconfluent TNF 6.8 cells were labeled with <sup>35</sup>S-cysteine as described above. The cells were rinsed with Buffer A (10 mM K<sub>2</sub>PO<sub>4</sub> [pH 7.0], 1 mM phenylmethylsulfonyl fluoride, PMSF, Sigma) and scraped into 1.5 ml Eppendorf microfuge tubes. The cells were sonicated with a Sonifier cell disruptor (Branson Sonic Power Co.), and checked for the extent of fragmentation by microscopic examination. The crude lysate was spun for 5 min at 2.7K rpm, 4°C, in an Eppendorf 5415 centrifuge (Brinkman). The supernatant was carefully withdrawn and spun in Beckman JA20 rotor at 11.5K rpm for 30 min (4°C). The supernatant was spun in a Beckman SW 50.1 rotor for 60 min (4°C), at 29K rpm. The membrane fraction pellet and cytosol supernatant were saved for immunoprecipitation analysis.

#### In Vitro Transcription

pGem-TNF14 and pGEM-TNF-LEAD were linearized with HindIII. Capped transcripts were prepared from these templates using T7 RNA polymerase and an in vitro transcription kit employing the conditions recommended by the manufacturer (Promega Biotec).

#### In Vitro Translation

<sup>35</sup>S-cysteine labeled polypeptides were synthesized from the in vitro TNF transcripts in the presence and absence of canine pancreatic microsomes (Promega) using a rabbit reticulocyte lysate translation kit (Promega) employing conditions recommended by the manufacturer.

#### Analysis of In Vitro Translation Products

In vitro translation products were analyzed as described by Teixeira et al. (1987). After in vitro translation, with or without microsomes, samples were diluted 5 fold with 100 mM Na<sub>2</sub>CO<sub>3</sub> (pH 11.5). After 30 min at 0°C, the samples were layered on top of a cushion of 200 mM sucrose (50 µl) containing 100 mM Na<sub>2</sub>CO<sub>3</sub> (pH 11.5), 85 mM potassium acetate, 1.8 mM magnesium acetate, and centrifuged for 8 min in a Beckman Airfuge A-100/30 rotor at 30 psi. The resulting pellets and supernatants were neutralized with 1 M acetic acid, and subjected to immunoprecipitation as described below.

Products of translations in the absence or presence of microsomes were incubated for 1 hr on ice, with or without 100 µg/ml proteinase K (Boehringer Mannheim). The proteolytic digestions were arrested by adjustment to 2 mM PMSF and subjected to immunoprecipitation as described below.

#### Immunoprecipitation

<sup>35</sup>S-labeled samples were immunoprecipitated with appropriate antisera following conditions described previously (Kriegler and Botchan, 1983). For competition experiments, 50 µg of cold recombinant TNF (Cetus) was added to appropriate samples before incubation with antisera.

#### Western Analysis

Samples were resolved on 12% SDS-PAGE and electrophoretically transferred to nitrocellulose membranes (Towbin et al., 1979). Filters were blocked and reacted with antibody, the bound antibody was detected either by using <sup>125</sup>I-Protein A followed by autoradiography or detected nonisotopically as described previously (Wong et al., 1986).

#### Radioiodination

Intact cells were radioiodinated in 24-well plates with <sup>125</sup>I and enzymebeads (BioRad) according to the manufacturer's instructions.

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*uppers of CPS and PANA*

*clap upon tissue of 101X*

## Production and Secretion of Tumor Necrosis Factor $\alpha$ in Endotoxin-treated Mono Mac 6 Cells Are Dependent on Phorbol Myristate Acetate\*

(Received for publication, May 21, 1992)

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Lipopolysaccharide (LPS, endotoxin) is a potent stimulator of tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) synthesis and secretion in mouse macrophage tumor cells (Golenbock, D. T., Hampton, R. Y., Qureshi, N., Takayama, K., and Raetz, C. H. R. (1991) *J. Biol. Chem.* 266, 19490-19498). In contrast, addition of LPS (10 ng/ml) to human monomyelocytic (Mono Mac 6) cells induces very little production of TNF $\alpha$ , as judged by immunoassay of the growth medium. When 30 ng/ml 4- $\beta$ -phorbol-12-myristate 13-acetate (PMA) is added together with LPS, large amounts of TNF $\alpha$  are secreted. PMA alone is inactive. Maximal TNF $\alpha$  levels in the medium are achieved at 1 ng/ml of LPS. Protein kinase C inhibitors, such as H7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine), staurosporine, and sphingosine, reduce TNF $\alpha$  secretion stimulated by PMA. The effect of PMA has been investigated at each stage of TNF $\alpha$  biogenesis. Treatment of Mono Mac 6 cells with LPS alone results in rapid, transient, and full expression of TNF $\alpha$  mRNA. Concomitant addition of PMA does not increase TNF $\alpha$  mRNA synthesis any further, but it prolongs the half-life of TNF $\alpha$  mRNA about 3-fold. However, mRNA stabilization does not account for the striking effect of PMA on TNF $\alpha$  secretion. Analysis of TNF $\alpha$  synthesis and secretion by immunoprecipitation indicates that LPS alone is fully effective in stimulating the formation of the intracellular 26-kDa TNF $\alpha$  precursor. LPS alone is not sufficient to allow processing of the precursor and secretion of mature 17-kDa TNF $\alpha$ . The rate of TNF $\alpha$  secretion observed immediately after the addition of PMA to LPS-pretreated cells is similar to the maximum rate from LPS/PMA-treated cells, but without the lag observed in cells after being exposed to LPS and PMA simultaneously. In summary, PMA is required for the completion of TNF $\alpha$  precursor processing and secretion in LPS-treated human Mono Mac 6 cells, whereas murine RAW cells are able to complete the terminal steps of TNF $\alpha$  processing in the absence of PMA.

acterized by its ability to cause the lysis of tumor cells (1), is a multifunctional cytokine (2), capable of influencing the growth, differentiation, and function of a broad range of cells (3). TNF $\alpha$  is responsible for some of the symptoms associated with endotoxin-induced shock and cachexia (2). TNF $\alpha$  is produced not only by activated macrophages (4) but also by other activated cells, including T cells (5), B cells (6), and NK cells (7). Bacterial lipopolysaccharide (LPS, endotoxin) is an essential component of the Gram-negative outer membrane (8), and it is a potent stimulus for TNF $\alpha$  production. Depending on the system, phorbol esters, viruses, and certain antibodies or antigens directed against T and/or B lymphocyte, surface receptors can also stimulate TNF $\alpha$  synthesis.

Secreted human TNF $\alpha$  is a 17-kDa polypeptide. Cloning of a full-length cDNA has revealed that TNF $\alpha$  is synthesized as a proprotein (26 kDa) with an N-terminal extension of 76 amino acid residues (9, 10). This presequence is not cleaved in the rough endoplasmic reticulum, like a typical signal sequence, but it may function to anchor the TNF $\alpha$  precursor polypeptide in the plasma membrane (11-13). The soluble 17-kDa form is derived from the 26-kDa membrane precursor by a specific proteolytic cleavage (12, 14, 15).

In most systems, TNF $\alpha$  production is regulated at the mRNA level. For instance, it has been shown that PMA induces TNF $\alpha$  synthesis in the monoblast cell line U937, in the myeloblast cell line ML-1 (16), or in human monocytes (17) by stimulating transcription of the TNF $\alpha$  gene and by posttranscriptional stabilization of the TNF $\alpha$  mRNA. Likewise, the down-regulation of TNF $\alpha$  expression observed during prolonged exposure of cells to LPS, PMA, or TNF $\alpha$  takes place at the mRNA level (18-20). Translational and post-translational regulation of TNF $\alpha$  synthesis have not received much attention, with the exception of a specific enhancement of the translation of TNF $\alpha$  mRNA by LPS in thioglycollate-elicited mouse macrophages (21).

In this study we describe a novel mechanism for the post-translational regulation of TNF $\alpha$  formation. Using the human monomyelocytic cell line, Mono Mac 6 (22), we show that PMA is required together with LPS to induce mature TNF $\alpha$  production. The expression of TNF $\alpha$  mRNA and the synthesis of the 26-kDa TNF $\alpha$  precursor are triggered normally by LPS, whereas PMA alone has no effect at either the transcriptional and translational levels in these cells. Instead, PMA is required for the processing and secretion of the precursor. This differs from previous observations with murine RAW 264.7 macrophage-like cells in which LPS addition alone (14, 23) is sufficient to cause TNF $\alpha$  synthesis and secretion.

### EXPERIMENTAL PROCEDURES

**Reagents**—Unless otherwise stated, all chemicals used are from Sigma. Murine monoclonal antibody F-12 against human TNF $\alpha$  was

Tumor necrosis factor  $\alpha$  (TNF $\alpha$ ),<sup>1</sup> a protein initially char-

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<sup>1</sup> The abbreviations used are: TNF $\alpha$ , tumor necrosis factor  $\alpha$ ; LPS, lipopolysaccharide; PMA, 4- $\beta$ -phorbol-12-myristate 13-acetate; PKC, protein kinase C; PBS, phosphate-buffered saline; FBS, fetal bovine serum; ACT D, actinomycin D; IgG, immunoglobulin G; H7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine; RIA, radioimmunoassay.

purchased from Olympos Immunochemicals (Lake Success, NY). Armenian hamster monoclonal antibody TN3-19.12 (24) against murine TNF $\alpha$  was a gift of Dr. D. E. MacIntyre (Merck Research Laboratories, Rahway, NJ). Rabbit antisera against human TNF $\alpha$ , rabbit anti-murine TNF $\alpha$ , and recombinant human and murine TNF $\alpha$  standards were purchased from Genzyme (Boston, MA). Preimmune rabbit IgG and rabbit IgG against human TNF $\alpha$  used in immunoprecipitation experiments were from Endogen (Boston, MA). Affinity-purified  $^{125}$ I-labeled goat anti-rabbit F(ab)'2 immunoglobulin fragments and carrier-free [ $^{32}$ P]orthophosphoric acid (8500–9120 Ci/mmol) were purchased from Du Pont-New England Nuclear. [ $^{35}$ S]Cysteine (1300 Ci/mmol) and [ $\alpha$ - $^{32}$ P]dCTP (3000 Ci/mmol) were from Amersham Corp. The nick translation kit was from Boehringer Mannheim. A human  $\beta$ -actin DNA probe (2 kb) was purchased from Clontech (Palo Alto, CA). A human TNF $\alpha$  DNA probe (0.607 kb) was a gift of Dr. M. J. Tocci (Merck Research Laboratories). Goat serum was purchased from Cedarlane Laboratories (Hornby, Ontario), and protein A-agarose was from GIBCO-Bethesda Research Laboratories.

Deep rough (Re) (8) LPS from the mutant, *Salmonella minnesota* R595, was prepared as described previously (25) and was provided by Dr. K. Takayama (Department of Bacteriology, University of Wisconsin, and Mycobacteriology Research Laboratory, William S. Middleton Memorial Veterans Hospital, Madison, WI). This preparation was used in all experiments as "LPS." Lipid IV $_A$ , a bioactive precursor of LPS (8), was purchased from ICN (Cleveland, OH). The LPS antagonist, *Rhodobacter sphaeroides* lipid A (RSLA), was provided by Drs. N. Qureshi and K. Takayama (Department of Bacteriology, University of Wisconsin).

**Cell Lines and Culture Methods**—Murine RAW 264.7 macrophage-like cells and L929 fibroblasts were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Mono Mac 6 cells were obtained from Dr. H. W. L. Ziegler-Heitbrock (Institute of Immunology, Munich, Germany). RAW 264.7 cells were grown as described previously (26) in Ham's F-12 medium (Whittaker Bioproducts, Walkersville, MD), supplemented with penicillin G (50 units/ml), streptomycin sulfate (50  $\mu$ g/ml, Sigma cell culture reagent grade), and 10% fetal bovine serum (FBS, Hyclone Laboratories). This medium (used also for all experiments with RAW 264.7) is referred to as F12/FBS. In general, RAW 264.7 cells were plated at  $0.5 \times 10^6$  cells/well in 24-well tissue culture plates in F-12/FBS and placed at 37 °C overnight to allow cell attachment prior to the initiation of stimulation. L929 fibroblasts, as adhered monolayers in 100-mm tissue culture dishes, and Mono Mac 6 cells (in suspension) were grown in RPMI 1640 medium (Whittaker Bioproducts), supplemented with antibiotics and FBS, as indicated above for F-12 medium, and with 1  $\times$  nonessential amino acids (GIBCO). This growth medium is referred to as complete medium. Before experiments, Mono Mac 6 cells were centrifuged ( $300 \times g$ , 5 min) and resuspended in complete medium at a density of  $2.5 \times 10^6$  cells/ml. All components used in these media were purchased as "low LPS" reagents. The different cell lines were grown at 37 °C in a 5% CO $_2$  atmosphere.

**Cell Stimulation and Labeling**—Dispersions of LPS, lipid IV $_A$ , and RSLA, prepared as 1 mg/ml stocks in endotoxin-free PBS (Whittaker Bioproducts, Walkersville, MD), were stored at -20 °C. Immediately before use, these stock solutions were thawed and sonicated for 2 min in a water bath sonicator (Laboratory Supplies Inc., Hicksville, NY) and then serially diluted in PBS to 100-fold concentrates.

The  $^{35}$ S labeling procedure of Mono Mac 6 cells was carried out in cysteine-free RPMI 1640 medium (GIBCO) containing 5% (v/v) dialyzed serum. Before labeling, the cells were washed twice with PBS and incubated for 30 min in the cysteine-free RPMI 1640 medium. The cells (approximately  $2.5 \times 10^6$  cells/ml) were then labeled in the same medium (referred to as "labeling medium") in the presence of 10  $\mu$ Ci/ $1 \times 10^6$  cells of [ $^{35}$ S]cysteine for the times indicated in the presence of the stimuli. The labeling procedure was terminated by removal of the labeling medium, followed by two washes with ice-cold PBS. For pulse-chase experiments, cells, at a density of  $2.5 \times 10^6$  cells/ml, were incubated in the labeling medium for various times and then washed twice with PBS and resuspended at the same density in complete medium. Radiolabeling of the cells with  $^{32}$ P was achieved by incubating the cells, after a pre-equilibration time (30 min) in phosphate-free RPMI 1640 medium (GIBCO), in a fresh phosphate-free RPMI 1640 medium with 250  $\mu$ Ci/ml of [ $^{32}$ P]orthophosphoric acid and stimulants for 50 minutes, as indicated.

At the end of these experiments, the culture supernatants were removed, and the cells were washed twice at 4 °C with PBS and

solubilized for 30 min at 4 °C in 100  $\mu$ l of "lysis buffer" per  $1 \times 10^6$  cells, consisting of 50 mM Tris-HCl, pH 7.4, 0.1 M NaCl, 1% Triton X-100, 5 mM EDTA, 0.02% sodium azide, 0.1 mM phenylmethanesulfonyl fluoride, and 1  $\mu$ M pepstatin. To remove insoluble material, cell lysates were centrifuged  $12,000 \times g$  for 15 min. The culture supernatants were also centrifuged ( $12,000 \times g$  for 15 min), and 50  $\mu$ l of  $10 \times$  concentrated lysis buffer (see above) was added per 500  $\mu$ l of culture supernatant.

**L929 Cytotoxicity Assay**—For assaying TNF $\alpha$  bioactivity, L929 cells, at a density of  $1 \times 10^5$  cells/well, were plated in 96-wells plates and incubated at 37 °C for 24 h in complete medium. Then the cells were washed, and samples to be tested were diluted from 1:2 to 1:128 in complete medium and were added to cells in the presence of actinomycin D (5  $\mu$ g/ml). The cells were incubated for 20 h, and the degree of cytotoxicity was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide dye reduction method (27). For this purpose, 10  $\mu$ l of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide solution (5 mg/ml) was added, and after another 4-h incubation at 37 °C, the resulting formazan crystals were dissolved in 10% SDS in 0.02 N HCl. Optical densities were measured at 570 nm (test wavelength) and 630 nm (reference wavelength) by using an automated plate reader (Bio-Tek Instruments). Recombinant TNF $\alpha$  was used as a standard for determination of the quantity of TNF $\alpha$  contained in the experimental samples. All samples were tested in triplicate.

**TNF $\alpha$  Radioimmunoassay**—TNF $\alpha$  production by cultured cells was quantitated by assaying TNF $\alpha$  immunoreactivity in the culture medium using a capture immunoassay similar to that described previously (24), but modified by S. D. Wright (The Rockefeller University, New York, NY) for 72-well Terasaki plates and radiodetection. The procedure was carried out exactly as described previously (23, 24) and was the same for the measurement of human or murine TNF $\alpha$ , except that the appropriate antibody and recombinant TNF $\alpha$  were used depending on the species. Briefly, the wells of Terasaki plates, first coated with monoclonal antibody anti-murine TNF $\alpha$  TN3-19-12 (or with monoclonal antibody anti-human TNF $\alpha$  F12), were then incubated overnight at 4 °C with PBS, 2% goat serum, 1 mg/ml bovine serum albumin, and 0.02% sodium azide. Then 10  $\mu$ l/well of experimental medium or recombinant murine (or human TNF $\alpha$ ) standard was added to the wells. After an incubation of 1 h at 22 °C and extensive washes, polyclonal rabbit anti-murine (or anti-human) antiserum was added. The plates were incubated 1 h at 22 °C and then washed. Next 10  $\mu$ l of  $^{125}$ I-labeled goat anti-rabbit F(ab)'2 immunoglobulin fragments (1  $\mu$ g/ml) were pipetted into each well. After 1 h of incubation, the plates were extensively washed, and individual wells cut from the plates were counted in a  $\gamma$  counter. The data were converted to ng/ml by use of a standard curve based on recombinant TNF $\alpha$ .

**Immunoprecipitation and Gel Electrophoresis**—To cell lysates or culture supernatants was added preimmune rabbit IgG or rabbit IgG against human TNF $\alpha$  at a final concentration of 40  $\mu$ g/ml. After an incubation of 18 h at 4 °C, 80  $\mu$ l of a 50% protein A-agarose bead suspension was added, and the incubation was performed at 4 °C for 2 h, with gentle agitation. The beads were then washed three times with lysis buffer and two times with PBS. Electrophoresis sample buffer (20 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 4% 2-mercaptoethanol, 0.04% bromophenol blue) was added to the washed beads, and the mixture was heated at 95 °C for 5 min. The samples were loaded onto a 12% polyacrylamide gel as described previously. Gels were fixed with 10% acetic acid, 40% methanol, treated with Amplify (Amersham Corp.), dried, and exposed to Kodak XAR-5 film at -80 °C. In some experiments the dried gels exposed to Phosphor Screens were scanned by Phosphorimager (Molecular Dynamics).

**Northern Blot Analysis**—Total RNA of Mono Mac 6 cells was isolated with RNazol (Cinna/Biotech Laboratories International Inc., Friendswood, TX), a modification of the guanidinium-phenol-chloroform method (28). For Northern blot analysis, 20  $\mu$ g of total RNA was electrophoresed on a 1.2% agarose (w/v) gel, transferred onto a Hybond-N membrane (Amersham Corp.), and hybridized with a nick-translated  $^{32}$ P-labeled human TNF $\alpha$  probe. Control hybridizations were done with a  $^{32}$ P-labeled human  $\beta$ -actin DNA probe, using the same blots after washing in 0.1% SDS at 75 °C for 10 min. TNF $\alpha$  mRNA (1.6 kb) was quantitated by densitometry with an LKB-XL laser photodensitometer and normalized to  $\beta$ -actin mRNA signals (2 kb).





## RESULTS

**RAW 264.7 and Mono Mac 6 Cells Differ in Their Ability to Secrete TNF $\alpha$  in Response to LPS**—The ability of the two cell lines, murine RAW 264.7 and human Mono Mac 6, to synthesize and secrete TNF $\alpha$  in response to LPS was examined. After stimulation for 5 h with as little as 0.01 ng/ml of LPS, RAW 264.7 cells secreted large amounts of TNF $\alpha$  (Fig. 1A). Maximum TNF $\alpha$  secretion (~30 ng/ml) was obtained at an LPS concentration of 1 ng/ml. The simultaneous addition of PMA (30 ng/ml) to RAW 264.7 cells treated with LPS did not further increase LPS-induced TNF $\alpha$  secretion, as shown in Fig. 1A. In contrast, very little TNF $\alpha$  (<0.5 ng/ml) was detected in the culture medium of Mono Mac 6 cells treated with LPS alone (Fig. 1B). However, when PMA (30 ng/ml) was added at the same time as LPS, Mono Mac 6 cells secreted large amounts of TNF $\alpha$  (~20 ng/ml). In the presence of PMA, the dose-dependent increase of secreted TNF $\alpha$  in response to LPS in Mono Mac 6 cells was very similar to that observed with RAW 264.7 cells. The minimum concentration needed to induce significant production of TNF $\alpha$  was 0.01 ng/ml LPS, and the optimal concentration of LPS was 1 ng/ml. The amount of TNF $\alpha$  secreted in the presence of LPS in combination with PMA always exceeded that seen in absence of PMA by 10–50-fold. The addition of PMA alone had no detectable effect on TNF $\alpha$  production, either in RAW 264.7 or in Mono Mac 6 cells (Fig. 1).

As shown in Fig. 2A, the minimum PMA concentration needed to induce the LPS-stimulated secretion of TNF $\alpha$  in Mono Mac 6 cells was just above 1 ng/ml. At 10 ng/ml of PMA, the stimulation reached a plateau. We have shown previously that another human monocytic cell line, THP-1 (25, 30), is poorly responsive to LPS. THP-1 cells need to be pretreated with PMA and allowed to differentiate to develop the ability to respond to LPS. Although the ability of PMA to stimulate TNF $\alpha$  secretion by Mono Mac 6 cells in the presence of LPS is evident without preincubation, we asked whether PMA pretreatment of Mono Mac 6 cells could lead to even higher stimulation of TNF $\alpha$  secretion by LPS. The cells were incubated with 30 ng/ml of PMA for 0–48 h before the addition of LPS. As shown in Fig. 2B, a preincubation for 1 h with PMA before the addition of LPS was as effective as concurrent addition. After preincubation for as little as 2 h, a significant unexpected decrease of TNF $\alpha$  secretion in response to LPS was observed. Moreover after preincubation with PMA for 24 h, the stimulation of TNF $\alpha$  secretion by LPS was fully abolished. Thus, in contrast to THP-1 cells, Mono Mac 6 cells do not need to be precultured with PMA to

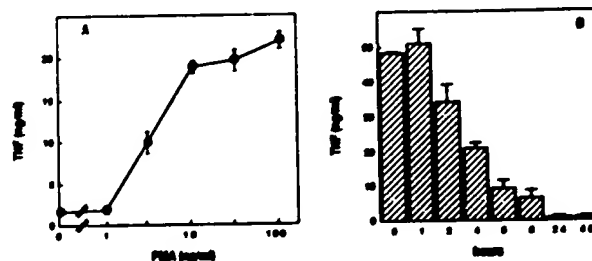


FIG. 2. Dose dependence and time course effect of PMA on TNF $\alpha$  release by Mono Mac 6 cells. A, Mono Mac 6 cells at a density of  $2.5 \times 10^5$ /ml were incubated in complete medium supplemented with LPS at 10 ng/ml and varying concentrations of PMA, as indicated, during 5 h at 37 °C. The culture supernatants were assayed for TNF $\alpha$  immunoreactivity by RIA. B, Mono Mac 6 cells were precultured with PMA at 30 ng/ml for the indicated times and then washed, resuspended at the same density as above in complete medium, and stimulated with LPS at 10 ng/ml for 5 h in the presence of 30 ng/ml of PMA at 37 °C. The media were assayed for TNF $\alpha$  immunoreactivity by RIA. Values represent the mean concentrations from duplicate samples  $\pm$  range.

show significant TNF $\alpha$  secretion in response of LPS.

**Alternative Assays for TNF $\alpha$** —A TNF $\alpha$  bioactivity assay was performed to ensure that the low levels of TNF $\alpha$  in culture supernatants from cells treated with LPS in the absence of PMA were not due to a lack of recognition of TNF $\alpha$  secreted under these conditions by the monoclonal antibody used in the radioimmunoassay. For instance, in LPS-treated cells and in PMA/LPS-treated cells, the secreted TNF $\alpha$  species might be different. The amounts of TNF $\alpha$  detected in culture supernatants from control, LPS-treated, or PMA/LPS-treated cells were 0, 0.5, and 8 ng/ml, respectively, when assayed by the cytotoxicity assay on L929 cells, in agreement with the results determined from the same culture supernatants by radioimmunoassay (data not shown).

To exclude the possibility that the low levels of TNF $\alpha$  secretion in response to LPS alone might have resulted from the production of an inhibitor that blocked the TNF $\alpha$ -monoclonal antibody interaction or the cytotoxic activity of TNF $\alpha$ , equal volumes of culture supernatants from LPS-treated cells and PMA/LPS-treated cells were mixed and assayed for the presence of TNF $\alpha$  by radioimmunoassay. The determination of TNF $\alpha$  in the culture medium was not obscured under these conditions (data not shown).

**Effects of Protein Kinase C Inhibitors**—PMA is a potent activator of protein kinase C (PKC). In order to ascertain whether protein kinase C was playing a role in the regulation of PMA-stimulated TNF $\alpha$  secretion in Mono Mac 6 cells, different inhibitors of PKC were examined. The results of Table I show that the effect of PMA on LPS-stimulated TNF $\alpha$  release was abolished by H7, staurosporine, and D-sphingosine, suggesting that the effect of PMA on TNF $\alpha$  secretion is actually mediated by the activation of a PKC. These different PKC inhibitors also blocked LPS stimulation of TNF $\alpha$  secretion in RAW 264.7 cells (Table I), although PMA alone had no stimulatory effect on TNF $\alpha$  release in RAW 264.7 cells. Nevertheless, a 24-h preincubation of RAW cells with 30 ng/ml of PMA did not inhibit the LPS-stimulated TNF $\alpha$  release, as it did in Mono Mac 6 cells.

**LPS-induced TNF $\alpha$  mRNA Expression Is Independent of PMA in RAW and Mono Mac 6 Cells**—PMA is known to increase TNF $\alpha$  mRNA expression in certain human myelomonocytic cell lines, such as ML-1, U937, and KG-1 (16), or in isolated human monocytes (17). In order to know whether PMA acts at the level of mRNA expression in Mono Mac 6

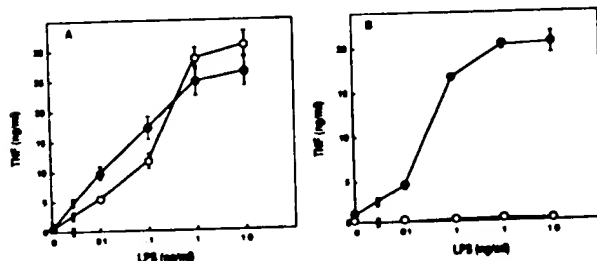


FIG. 1. Combined effect of LPS and PMA on TNF $\alpha$  release by Mono Mac 6 cells. RAW 264.7 cells at a density of  $0.5 \times 10^5$ /well (in 24 well-plates) in F-12/FBS (A) and Mono Mac 6 cells at a density of  $2.5 \times 10^5$ /ml in complete medium (B) were incubated at 37 °C with varying concentrations of LPS as indicated in the presence (●) or absence (○) of PMA (30 ng/ml) during 5 h. Then the culture supernatants were assayed for TNF $\alpha$  immunoreactivity by RIA. Values represent the mean concentrations from duplicate samples  $\pm$  range.

cells, we studied TNF $\alpha$  mRNA production in response to LPS in the presence or absence of PMA. The dose-response relationships of induced TNF $\alpha$  mRNA expression after 90 min of incubation are presented in Fig. 3. TNF $\alpha$  mRNA levels were maximal at 10 ng/ml of LPS (as observed above for secreted TNF $\alpha$ ), but in contrast to the situation with secreted TNF $\alpha$ , PMA had no effect on TNF $\alpha$  mRNA expression (Fig. 3, A and C). The expression of TNF $\alpha$  mRNA in RAW 264.7 cells was fully induced by 10 ng/ml of LPS, and PMA did not increase the TNF $\alpha$  mRNA expression in RAW 264.7 cells any further (data not shown).

The desensitization observed with Mono Mac 6 cells after a long-term preincubation with PMA with respect to TNF $\alpha$  secretion (Fig. 2B) was investigated at the level of TNF $\alpha$  mRNA expression. After a 24-h treatment of Mono Mac 6 cells with PMA (30 ng/ml), the expression of the TNF $\alpha$  mRNA was still markedly induced by LPS, as in untreated cells (data not shown). However, only a little TNF $\alpha$  (0.35  $\pm$

0.03 ng/ml) was actually secreted by the pretreated cells in comparison with the large amount of TNF $\alpha$  secreted by control cells (22  $\pm$  1.5 ng/ml) treated with LPS and PMA concurrently. Thus, long-term preincubation with PMA has no effect on the ability of the TNF $\alpha$  gene to respond to LPS, but rather, prolonged PMA pretreatment appears to block a later stage of the TNF $\alpha$  biosynthetic pathway.

**PMA Enhances TNF $\alpha$  mRNA Stability**—The time course study (Fig. 3, B and D) shows that the addition of LPS at 10 ng/ml led to a quick and transient expression of TNF $\alpha$  mRNA. Within 30 min, 50% of the TNF $\alpha$  mRNA was induced, and by 60 min maximal expression was achieved. After 90 min, the steady-state level of TNF $\alpha$  mRNA began to decrease, and TNF $\alpha$  mRNA disappeared almost completely after 6 h of incubation with LPS. The addition of PMA (which had no detectable effect by itself on the induction of TNF $\alpha$  mRNA at early times) led to an increased steady-state level of TNF $\alpha$  mRNA at later times. The higher level of TNF $\alpha$  mRNA observed in the presence of PMA apparently resulted from an increase of TNF $\alpha$  mRNA stability, because as shown in Fig. 3, A and C, PMA alone was unable to induce TNF $\alpha$  mRNA expression. To investigate this issue further, we measured the half-life of TNF $\alpha$  mRNA using actinomycin D (5  $\mu$ g/ml) to inhibit RNA synthesis. As indicated in Fig. 4, in the presence of PMA, the half-life of TNF $\alpha$  mRNA was increased. The values determined for TNF $\alpha$  mRNA disappearance in cells treated or not treated with PMA together with actinomycin D were 33 and 12 min, respectively. The addition of PMA 1 h before actinomycin D led to an even greater stabilization of TNF $\alpha$  mRNA ( $t_{1/2}$  = 48 min). Thus, the somewhat higher induction of TNF $\alpha$  mRNA at later times in the presence of PMA may be explained by stabilization of TNF $\alpha$  mRNA. However, this modest stabilization of TNF $\alpha$  mRNA by PMA does not adequately explain the much greater effect of PMA on TNF $\alpha$  secretion.

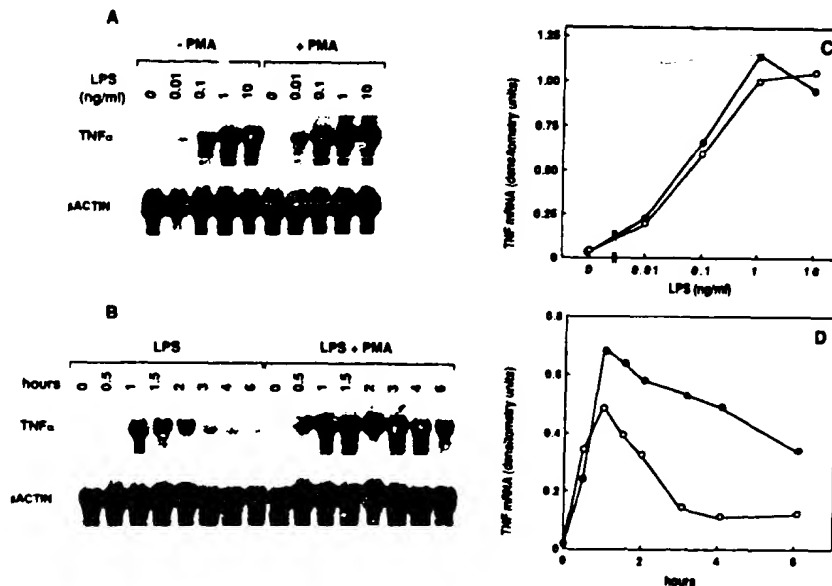
**LPS Antagonists Block LPS-induced mRNA Synthesis in Mono Mac 6 Cells**—As observed by Golenbock *et al.* (30) with human THP-1 cells and with human monocytes, lipid IV<sub>A</sub>, an underacylated disaccharide precursor of lipid A, is also a potent antagonist of LPS action on human Mono Mac 6 cells (data not shown). TNF $\alpha$  mRNA expression in LPS-treated

TABLE I  
Effect of various inhibitors of protein kinase C on LPS- or LPS/PMA-stimulated TNF $\alpha$  secretion

RAW 264.7 and Mono Mac 6 cells were incubated in F12/FBS and complete medium, respectively, for 15 min with the different PKC inhibitors described. Then LPS (10 ng/ml) was added to RAW 264.7 cells, and LPS (10 ng/ml) and PMA (30 ng/ml) were added to Mono Mac 6 cells. After 5 h of incubation, the culture supernatants were collected and analyzed for TNF $\alpha$  immunoreactivity by RIA. The values represent the mean concentrations from duplicates samples  $\pm$  range and are representative of two separate experiments.

Conditions	TNF $\alpha$	
	Mono Mac 6 cells	RAW 264.7 cells
	ng/ml	
No stimulus	0.30 $\pm$ 0.01	0.50 $\pm$ 0.01
Control (LPS/PMA or LPS)	17.09 $\pm$ 0.87	43.02 $\pm$ 2.05
H7 100 $\mu$ M	6.86 $\pm$ 0.36	21.30 $\pm$ 3.2
H7 200 $\mu$ M	3.04 $\pm$ 0.35	17.78 $\pm$ 0.27
H7 400 $\mu$ M	0.89 $\pm$ 0.05	7.63 $\pm$ 0.12
Staurosporine, 2 nM	1.85 $\pm$ 0.03	7.66 $\pm$ 0.5
Staurosporine, 20 nM	0.22 $\pm$ 0.09	4.38 $\pm$ 0.26
Sphingosine, 25 $\mu$ M	5.10 $\pm$ 0.01	37.17 $\pm$ 1.51
Sphingosine, 250 $\mu$ M	0.36 $\pm$ 0.34	0.89 $\pm$ 0.08

FIG. 3. TNF $\alpha$  mRNA expression in Mono Mac 6 cells after stimulation with LPS and PMA. A, Mono Mac 6 cells at a density of  $2.5 \times 10^6$ /ml were stimulated 2 h with varying concentrations of LPS in the presence or absence of PMA (30 ng/ml) in complete medium. Then total RNA was isolated and analyzed for TNF $\alpha$  mRNA content, as described under "Experimental Procedures." B, Mono Mac 6 cells at a density of  $2.5 \times 10^6$ /ml were stimulated by 10 ng/ml of LPS in the presence or absence of PMA (30 ng/ml) in complete medium at 37  $^{\circ}$ C. At the indicated times, total RNA was isolated and analyzed for TNF $\alpha$  mRNA content. C and D, the values represent the arbitrary units of TNF $\alpha$  mRNA contents of the cells after stimulation by LPS in the absence (O) or presence of PMA (●), obtained by densitometry scanning of Northern blot autoradiographs from A and B, respectively, and normalized to the  $\beta$ -actin signals.



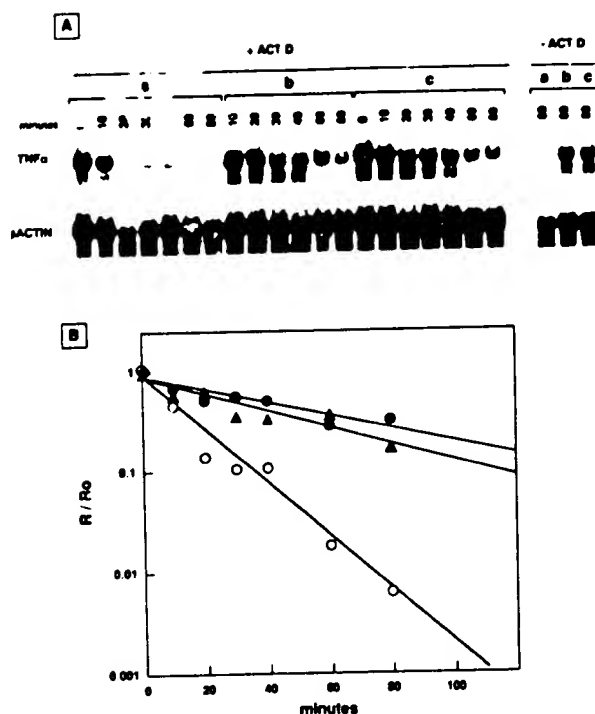


FIG. 4. Stabilization of TNF $\alpha$  mRNA by PMA. Mono Mac 6 cells were stimulated for 1 h with LPS in the absence (a and b;  $\circ$  and  $\Delta$ ) or presence of 30 ng/ml PMA (c;  $\bullet$ ). At time 0, 5  $\mu$ g/ml of actinomycin D (+ACT D;  $\circ$ ,  $\bullet$ ,  $\Delta$ ) or nothing (-ACT D) was added in the absence (a;  $\circ$ ) or presence (b and c;  $\bullet$ ,  $\Delta$ ) of 30 ng/ml PMA. At the indicated times, total RNA was isolated and analyzed for TNF $\alpha$  mRNA. The amount of TNF $\alpha$  mRNA was quantitated by densitometry of Northern blot autoradiographs (A) after normalization to  $\beta$ -actin signals. The ratio ( $R/R_0$ ) of the residual amount ( $R$ ) of TNF $\alpha$  mRNA determined at the times indicated to the initial amount of TNF $\alpha$  mRNA ( $R_0$ ) at time 0 is plotted in B.

cells and TNF $\alpha$  secretion from PMA/LPS-stimulated cells are both inhibited. Furthermore, the LPS antagonist, RSLA (8), also blocks the stimulatory effect of LPS on TNF $\alpha$  mRNA expression in human Mono Mac 6 cells (data not shown), as it does in RAW cells. Despite the differences in the physiology of these three cell lines, RSLA behaves as an antagonist in all instances, suggesting that the receptor(s) involved in the early stages of signal transduction are similar.

**A 26-kDa Protein Precursor of TNF $\alpha$  in LPS-treated Mono Mac 6 Cells.**—The PMA dependence of TNF $\alpha$  secretion in LPS-stimulated Mono Mac 6 cells might be the result of a regulatory effect of PMA on TNF $\alpha$  mRNA translation, on cellular TNF $\alpha$  protein stabilization, and/or the TNF $\alpha$  secretion process itself. To examine cellular TNF $\alpha$  protein synthesis in Mono Mac 6 cells, the cells were metabolically labeled with [ $^{35}$ S]cysteine and treated with LPS and PMA for 2.5 h. Next, cell lysates and culture supernatants were subjected to TNF $\alpha$  protein analysis by immunoprecipitation, as explained under "Experimental Procedures." The results of this analysis are presented in Fig. 5 and show that high levels of mature 17-kDa TNF $\alpha$  were observed only in media of LPS/PMA-treated cells (Fig. 5, lane 3), but not of LPS-treated cells (Fig. 5, lane 2).

In cell lysates a 26-kDa protein corresponding to the 26-kDa TNF $\alpha$  precursor was detected, irrespective of whether cells were stimulated by LPS or LPS/PMA (Fig. 5, lanes 8 and 9). These findings exclude an effect of PMA on glycosyl-

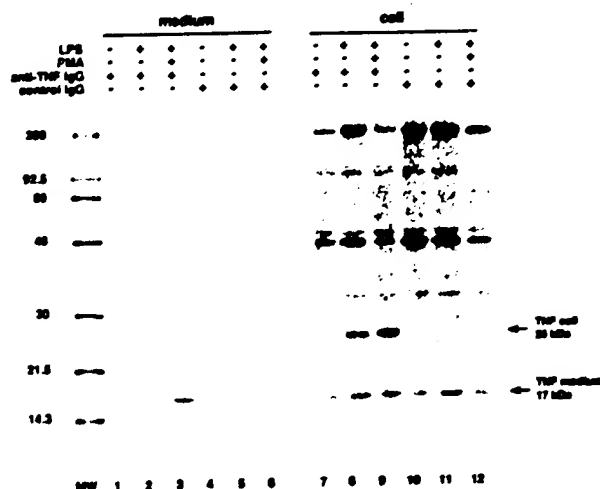


FIG. 5. Immunoprecipitation analysis of TNF $\alpha$  from Mono Mac 6 cell lysates and culture supernatants. Mono Mac 6 cells at a density of  $2.5 \times 10^6$ /ml were incubated for 2.5 h at 37 °C in the presence or absence of LPS (10 ng/ml), and with or without PMA (30 ng/ml) in the labeling medium, as indicated and described under "Experimental Procedures." Lysates from  $5 \times 10^6$  cells (lanes 7-12) or 1 ml of the culture supernatants (lanes 1-6) were immunoprecipitated with anti-human TNF $\alpha$  rabbit IgG (lanes 1-3 and 7-9) or with preimmune rabbit IgG (lanes 4-6 and 10-12) and analyzed by electrophoresis. The positions of molecular weight markers are shown on the left, and TNF $\alpha$  immunoreactive bands are identified by their apparent molecular weight at the right.

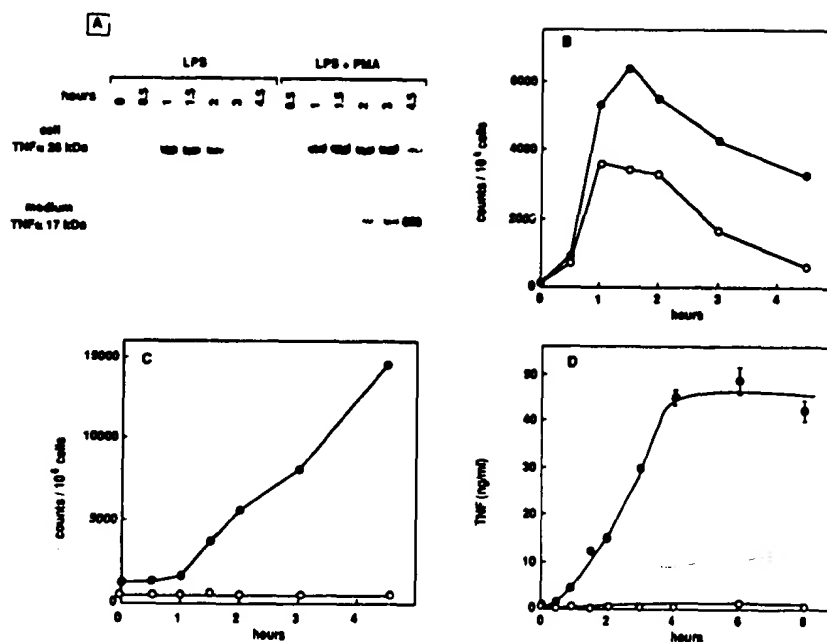
ation (14) or acylation (31) in these cells. Although a protein of 17 kDa was also detected in Mono Mac 6 cell lysates, this did not correspond to mature TNF $\alpha$ , because it was seen after precipitation with either immune antibody or preimmune antibody (lanes 7-9 and 10-12, respectively). The band at 17 kDa, as well as those at 200, 93, 51, 45, and 33 kDa detected in whole cell lysates, were probably precipitated by nonspecific interactions.

We could not exclude the possibility that treatment with PMA, an activator of PKC, stimulated the phosphorylation of TNF $\alpha$ , although no one has described the phosphorylation of TNF $\alpha$ . No [ $^{32}$ P]-labeled TNF $\alpha$  was detected in lysates of [ $^{32}$ P]orthophosphate-labeled Mono Mac 6 cells under any of the conditions of stimulation tested, indicating that TNF $\alpha$  was not phosphorylated, even after stimulation with LPS/PMA (data not shown).

**Time Course of TNF $\alpha$  Synthesis and Secretion.**—We next examined the time course of TNF $\alpha$  protein expression in response to LPS and PMA stimulation. As expected, the mature 17-kDa TNF $\alpha$  could be detected only in the culture supernatants of the cells treated with LPS (10 ng/ml) in combination with PMA (30 ng/ml) (Fig. 6, A and C). The 17-kDa TNF $\alpha$  appeared in the medium after a lag of 60 min and accumulated during the time of the stimulation. A quantification of the secreted TNF $\alpha$  by radioimmunoassay as function of time (Fig. 6D) showed that the maximal secretion of TNF $\alpha$  was reached between 4 and 6 h of stimulation by LPS and PMA.

The time course of the induction of the intracellular 26-kDa TNF $\alpha$  in response to LPS with or without PMA is shown Fig. 6, A and B. After 30 min of incubation with 10 ng/ml of LPS alone or in presence of 30 ng/ml of PMA, the 26-kDa TNF $\alpha$  was already expressed and reached a maximum after 90 min of stimulation. The kinetics of the expression of the cellular 26-kDa TNF $\alpha$  protein (Fig. 6, A and B) were very

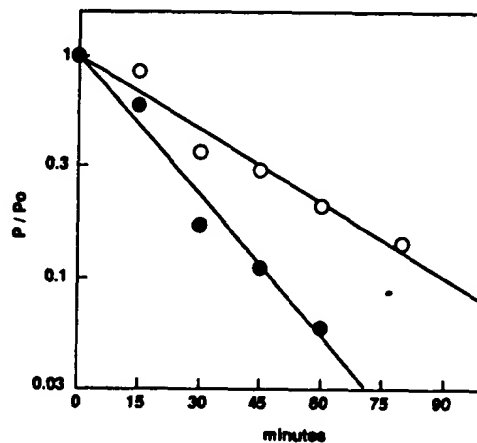
**FIG. 6. Immunoprecipitation analysis of TNF $\alpha$  secretion and function of time.** A, C, Mono Mac 6 cells at a density of  $2.5 \times 10^6$ /ml were incubated in the labeling medium at 37°C with LPS (10 ng/ml) in the presence (●) or absence (○) of PMA (30 ng/ml). At the times indicated, cell lysates were prepared, and media were collected for immunoprecipitation of TNF $\alpha$  with anti-human TNF $\alpha$  IgG, as detailed under "Experimental Procedures." All samples were analyzed by SDS-polyacrylamide gel electrophoresis, and the quantification of cellular and secreted TNF $\alpha$  was determined by Phosphorimager analysis of the gels. The densitometry units obtained for the cellular TNF $\alpha$  and for the secreted TNF $\alpha$  are shown in curves B and C, respectively. D, Mono Mac 6 cells at a density of  $2.5 \times 10^6$ /ml were incubated in complete medium with LPS (10 ng/ml) in the presence (●) or absence (○) of PMA (30 ng/ml). At the times indicated, the media were collected and assayed for TNF $\alpha$  by RIA. Points and error bars represent the mean and range of duplicate samples.



similar under both conditions, but the levels of the 26-kDa protein were about 2-fold higher in presence of PMA than in its absence. This observation could be explained by the higher level of TNF $\alpha$  mRNA under these culture conditions (Fig. 3, B and D).

To exclude that PMA was also causing a stabilizing effect at the protein level, the half-life of the 26-kDa TNF $\alpha$  was examined in a pulse-chase experiment. The kinetics of disappearance of cellular 26-kDa TNF $\alpha$  during a chase initiated after a 45-min labeling period in the presence of 10 ng/ml LPS (analyzed by immunoprecipitation and electrophoresis) are shown in Fig. 7. The addition of PMA (30 ng/ml) during the chase period led to a faster disappearance of TNF $\alpha$  in cells (from  $t_{1/2} = 28$  min to  $t_{1/2} = 14$  min), probably by induction of TNF $\alpha$  secretion. As shown in Fig. 6, A and B, and in Fig. 7, the 26-kDa TNF $\alpha$  did not accumulate to a greater extent in the cells treated with LPS than with LPS/PMA. Given that the TNF $\alpha$  was not secreted into the extracellular medium during stimulation with LPS alone, intracellular degradation may be occurring to prevent further accumulation.

**PMA Has a Specific and Direct Effect on TNF $\alpha$  Secretion—**We investigated this last issue by examining the rate of secretion of TNF $\alpha$  under different incubation conditions. As indicated in the scheme at the bottom of Fig. 8, cells were preincubated for 1 h without any stimulants, with LPS alone, with PMA alone, or with both LPS and PMA. Next (referred to as time 0 in the experiment), PMA and/or LPS were added to various cultures, as indicated, and the resulting supernatants were analyzed for TNF $\alpha$  by radioimmunoassay after different times (Fig. 8). A lag of 60 min was observed before 17-kDa TNF $\alpha$  could be detected in the medium of untreated or PMA-pretreated cells, followed by the addition of LPS and PMA at time 0. This lag corresponded approximately to the time needed for full induction of TNF $\alpha$  mRNA and 26-kDa TNF $\alpha$  (Fig. 3, B and D, and Fig. 5, A and B, respectively). In contrast, as soon as PMA was added to the culture medium of LPS-pretreated cells, there was an immediate release of 17-kDa TNF $\alpha$ . Following the addition of PMA to LPS-pretreated cells, the TNF $\alpha$  secretion rate became identical to that ob-



**FIG. 7. Effect of PMA on turnover of [ $^{35}$ S]-labeled 26-kDa TNF $\alpha$  precursor.** Mono Mac 6 cells at a density of  $2.5 \times 10^6$ /ml were stimulated in complete medium with LPS (10 ng/ml) in the presence of 10  $\mu$ Ci of [ $^{35}$ S]cysteine,  $1 \times 10^6$  cells for 50 min. The cells were washed and then stimulated again in complete medium with LPS (10 ng/ml) in the absence (○) or presence (●) of PMA (30 ng/ml). Cell lysates were prepared at the times indicated (time 0 of the curve is time 0 of the chase). TNF $\alpha$  was immunoprecipitated and analyzed by SDS-polyacrylamide gel electrophoresis. The TNF $\alpha$  amounts were quantitated by Phosphorimager analysis. The ratio ( $P/P_0$ ) of the residual amount ( $P$ ) determined at the times indicated to the initial amount ( $P_0$ ) determined at time 0 is plotted.

served with cells preincubated in the presence of the combination of LPS and PMA (Fig. 8). These results indicate that the 26-kDa TNF $\alpha$  was synthesized normally in LPS-stimulated cells, but not secreted, as suggested by Figs. 5 and 6, and that it was susceptible to instantaneous processing and secretion in response to PMA specifically.

The TNF $\alpha$  secretion rate following PMA addition to LPS-pretreated cells was not higher than that of the LPS and PMA preincubated cells (Fig. 8), confirming that in cells stimulated by LPS alone, the 26-kDa protein did not accumulate to generate an extraordinarily large intracellular pool.

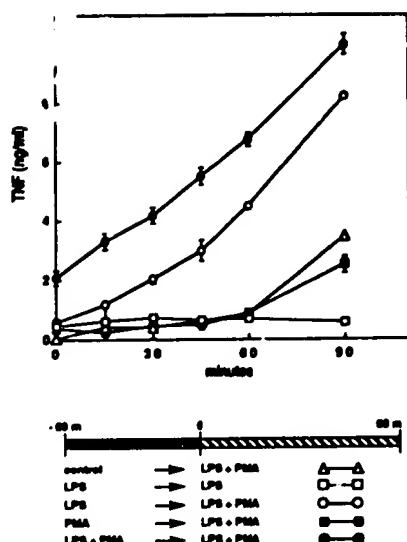


FIG. 8. PMA effect on the induction of TNF $\alpha$  secretion in Mono Mac 6 cells. Mono Mac 6 cells at a density of  $2.5 \times 10^6$ /ml were preincubated for 1 h in complete medium ( $\Delta$ ) supplemented with LPS (10 ng/ml) ( $\square$ ,  $\circ$ ), with PMA (30 ng/ml) ( $\blacksquare$ ), or with LPS and PMA ( $\bullet$ ). Then, referred as to time 0 on the curve, media were removed and replaced by a fresh complete medium, supplemented with LPS alone ( $\square$ ) or with LPS and PMA ( $\Delta$ ,  $\blacksquare$ ,  $\bullet$ ,  $\circ$ ). At the times indicated, media were collected and analyzed for TNF $\alpha$  immunoreactivity by RIA. Points and error bars represent the mean and range of duplicate samples.

It may be that TNF $\alpha$  secretion is coupled to translation. In LPS, as in LPS/PMA pretreated cells, the subsequent addition of cycloheximide at time 0 inhibited PMA-induced secretion of TNF $\alpha$  fully and immediately (data not shown in Fig. 8).

All these results taken together suggest that PMA facilitates the secretion of TNF $\alpha$  in Mono Mac 6 cells. In the presence of LPS alone, the synthesis of the 26-kDa TNF $\alpha$  precursor does occur, but may be accompanied by intracellular degradation to maintain a relatively low steady state pool. In the presence of PMA, this intracellular degradation is less likely to occur, because TNF $\alpha$  molecules are rapidly processed and secreted into the medium.

#### DISCUSSION

The stimulatory effects of PMA on macrophage and monocyte function have been studied extensively (16, 17, 32–37). Depending on the cell type, early activation events include the release of arachidonic acid (32), the phosphorylation of proteins (33), and the expression of genes, such as *c-fos* and *c-myc* (34). Later events include the stimulation of cytokine synthesis (35), particularly the production of TNF $\alpha$  (36, 37). In many instances PMA addition leads to increased TNF $\alpha$  gene transcription (16) and/or TNF $\alpha$  mRNA stabilization (17). LPS has similar effects on the expression of the TNF $\alpha$  gene in certain cell lines (38). But in other situations LPS primes cells for the ability to respond to PMA (52). In all these systems it has been difficult to distinguish cause from effect at a biochemical level.

In the present study we have documented a novel role for PMA in stimulating processing and secretion of TNF $\alpha$  in the human myelomonocytic cell line Mono Mac 6. These cells differ from most lines examined so far in that they are able to secrete large amounts of TNF $\alpha$  in response to LPS only when PMA is present simultaneously (Figs. 1 and 2). In

contrast, the mouse macrophage-like cell line, RAW 264.7 (Ref. 23 and Fig. 1), responds to LPS alone with a significant release of TNF $\alpha$ , irrespective of the presence or absence of PMA. Direct analysis of TNF $\alpha$  mRNA revealed that LPS was able to stimulate the induction of TNF $\alpha$  mRNA expression in Mono Mac 6 cells (Fig. 3), whether or not PMA was present. Unlike some monocytic human cell lines, which need preincubation with PMA to become responsive to LPS, such as U937 (38) or THP-1 cells (30), PMA was not necessary to induce LPS responsiveness in the Mono Mac 6 system. PMA actually caused the cells to lose their LPS responsiveness (see below).

Our data confirm that the Mono Mac 6 cell line has many of the phenotypic and functional characteristics of peripheral blood monocytes, as described by Ziegler-Heitbrock *et al.* (22). However, they claimed that Mono Mac 6 cells were able to secrete TNF $\alpha$  in response to LPS (19) alone, as judged by a cytotoxicity assay ( $^{51}\text{Cr}$  release from WEHI 164 cells). The TNF $\alpha$  observed by these workers probably corresponded to the small quantities that we detected by our radioimmunoassay (0.2–2 ng/ml) in the absence of PMA, since Ziegler-Heitbrock *et al.* (22) did not test the effect of simultaneous PMA and LPS addition. However, they did show that culturing the cells for 3 days with PMA inhibited LPS-stimulated TNF $\alpha$  secretion. They suggested that this reduction of TNF $\alpha$  secretion was due to suppression of TNF $\alpha$  mRNA expression. We found that preincubation with PMA for as little as 2 h rendered Mono Mac 6 cells significantly less responsive to LPS (Fig. 2) in terms of TNF $\alpha$  secretion and that this PMA-induced desensitization was maximal after 24 h. We demonstrated that PMA-induced desensitization to LPS was not occurring at the level of TNF $\alpha$  mRNA production, but rather, at a later step of TNF $\alpha$  processing and secretion. The PMA desensitization effect on Mono Mac 6 cells might be due to PKC down-regulation, as described previously (33, 39), but the concentration of PMA (30 ng/ml) we used was lower than that known to deplete PKC activity (1  $\mu\text{g}/\text{ml}$ ).

Several lines of evidence indicate that the stimulation of TNF $\alpha$  secretion somehow requires the action of PKC. Previous studies have demonstrated that the biologically active lipid moieties of lipopolysaccharide can activate PKC in extracts of RAW 264.7 cells (40). We found that PMA by itself did not stimulate measurable TNF $\alpha$  secretion or TNF $\alpha$  mRNA expression in RAW 264.7 cells and that it did not induce greater TNF $\alpha$  secretion or TNF $\alpha$  mRNA expression when added in combination with LPS. However, inhibitors of PKC blunted the ability of LPS to do so (Table I). These data suggest that, in RAW 264.7 cells, PKC function may be necessary but not sufficient for TNF $\alpha$  secretion. In contrast, addition of PMA is needed to induce TNF $\alpha$  secretion in LPS-treated Mono Mac 6 cells, suggesting that LPS by itself is not able to activate PKC in Mono Mac 6 cells (as has been reported for HL60 cells (41)). The ability of LPS to activate TNF $\alpha$  mRNA and intracellular TNF $\alpha$  precursor synthesis in Mono Mac 6 cells (in the absence of any effect of PMA by itself on TNF $\alpha$  mRNA expression) suggests that PKC activation is necessary at a very late step in TNF $\alpha$  biogenesis, possibly during secretion.

Nevertheless, it is generally believed that TNF $\alpha$  production stimulated by PMA is mediated by a PKC-dependent pathway operating at the level of TNF $\alpha$  mRNA synthesis in murine macrophages (42). In the human TNF $\alpha$  gene, PMA-responsive 5'-flanking sequences are present, although their exact function remains uncertain (43). The differences in the regulation of cytokine synthesis in commonly used cell types are significant and require further investigation.



In thioglycollate-elicited mouse macrophages it has been reported that the TNF $\alpha$  gene is actively transcribed, but is not translated, indicating that TNF $\alpha$  production is regulated primarily at the translational level in these cells (21, 44). Our experiments (Figs. 5-9) demonstrated that LPS alone could induce the full expression of the 26-kDa TNF $\alpha$  precursor protein but not the appearance of mature 17-kDa TNF $\alpha$  in the medium. We conclude that PMA-mediated regulation of TNF $\alpha$  production in Mono Mac 6 cells must occur at the posttranslational level. The fact that an immediate release of mature TNF $\alpha$  occurs after PMA addition to LPS pretreated cells is the best evidence that PMA affects a late stage in the processing of TNF $\alpha$ .

Phosphorylation is an important mechanism involved in transporting and targeting of newly synthesized proteins (45, 46). LPS activation of IL-1 synthesis is accompanied by the phosphorylation of IL-1 precursor molecule, facilitating the processing and/or release of mature IL-1 (47, 48). We have shown that the 26-kDa precursor TNF $\alpha$ , unlike the IL-1 precursor, is not phosphorylated under any of the conditions tested. This observation raises the question of why PKC activation by PMA is needed to induce TNF $\alpha$  secretion in Mono Mac 6 cells.

First, phosphorylation may play a role in the intracellular routing of TNF $\alpha$  precursor. The intracellular localization of TNF $\alpha$  remains unknown. It has been shown that TNF $\alpha$  is present in the Golgi apparatus of human monocytes and T lymphocytes (49). In murine macrophages (11), in human monocytes (50), and in a TNF $\alpha$  nonsecreting mutant cell line (13), TNF $\alpha$  was also found in the plasma membrane.

Second, phosphorylation may activate an enzyme responsible for the proteolysis of the precursor of TNF $\alpha$ , allowing its release into the medium. The suppression of TNF $\alpha$  secretion by the inhibitor *p*-toluenesulfonyl-L-arginine methyl ester has suggested the possibility that TNF $\alpha$  secretion is dependent upon the action of serine proteases (51).

In summary, the unusual effects of PMA on TNF $\alpha$  production that we have observed in human Mono Mac 6 cells should provide novel insights into the biochemical events involved in the processing and secretion of TNF $\alpha$ .

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# Cytotoxins (Tumour Necrosis Factor, Lymphotoxin and Others): Molecular and Functional Characteristics and Interactions with Interferons

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## ABBREVIATIONS

*The following abbreviations and synonyms will be used*

ADCC	= antibody-dependent cytotoxicity	IL-1	= interleukin 1
CHI	= cycloheximide	LPS	= bacterial lipopolysaccharides
CTX	= cytotoxins	LT	= lymphotoxin
DTH	= delayed-type hypersensitivity	mAb	= monoclonal antibody
HA	= haemagglutination units	MHC	= major histocompatibility complex
IFN	= interferon	MP	= mononuclear phagocytes

NK	= natural killer	PHA	= phytohaemagglutinin
NKCF	= natural killer cytotoxic factor	PMA	= 4 $\beta$ phorbol-12-myristate-13-acetate
PAGE	= polyacrylamide gel electrophoresis	SDS	= sodium dodecyl sulphate
PBMC	= peripheral-blood mononuclear leukocytes	TNF	= tumour necrosis factor
		VSV	= vesicular stomatitis virus

## I INTRODUCTION

Awareness of the anticellular activity of the interferons (IFNs), and of the existence of cytokines other than IFNs with anticellular activity, has developed in parallel. Initial studies on the anticellular effects of IFNs were with crude preparations (Pauker *et al.*, 1962). This raised doubts about whether IFN in these preparations did indeed mediate the observed effects, and not other, contaminating, cytokines. Conversely, the existence of cytokines, other than IFNs, with cytotoxic activity ('cytotoxins'—CTXs) has for quite a time been questioned in view of the fact that the crude preparations in which these mediators were detected. (Granger and Kolb, 1968; Ruddle and Waksman, 1968; Carswell *et al.*, 1975) did contain IFNs. With the purification of IFNs and, very recently, two CTXs—lymphotoxin and tumour necrosis factor (Aggarwal *et al.*, 1984, 1985; Haranaka *et al.*, 1985) it was confirmed that both the IFNs and the CTXs have their own anticellular activities. It was also observed that the two kinds of mediators may act in concert: their anticellular activities are synergistic; other effects of CTXs, besides their anticellular ones, are also potentiated by IFNs. Furthermore, IFNs promote the formation of CTXs, and CTXs can induce the formation of IFNs.

We are attempting now to throw light on the mechanisms which underlie these interrelationships of IFNs and CTXs and to understand their physiological significance. Our concepts on these subjects are still in a state of flux. In fact, even the terminology for the CTXs is still undefined and may have to remain so until we have a better idea of the extent of multiplicity and heterogeneity of the CTXs. In this review, I have tried to extract from the fragmentary information available as comprehensive an understanding as possible and to speculate on aspects in which there are still gaps in our knowledge. A suggestion made by Donald Metcalf (1975) may be appropriate to apply at this point: 'Remember that no matter how enthusiastic you may be about the significance of your observations, if your paper is re-read in 10 years' time, your original interpretation of the data will almost certainly be wrong, if not hilariously so.' It may take much less than 10 years in the field of CTX research.

This review focuses on those aspects of our knowledge of CTXs concerning the interrelationship between CTXs and IFNs. For a survey of other aspects

- PHA = phytohaemagglutinin  
 PMA = 4 $\beta$ phorbol-12-myristate-13-acetate  
 SDS = sodium dodecyl sulphate  
 TNF = tumour necrosis factor  
 VSV = vesicular stomatitis virus

## DUCTION

of the interferons (IFNs), and of the IFNs with anticellular activity, has the anticellular effects of IFNs were (Granger and Kolb, 1968; Ruddle *et al.*, 1975) did contain IFNs. With the addition of two CTXs—lymphotoxin and tumour necrosis factor (TNF) (Granger and Kolb, 1968; Ruddle *et al.*, 1975; Haranaka *et al.*, 1985) and the CTXs have their own anti-tumour activity. The two kinds of mediators may be synergistic; other effects of IFNs are also potentiated by IFNs. Furthermore, CTXs and CTXs can induce the

on the mechanisms which underlie CTXs and to understand their physiological effects are still in a state of flux. The definition of CTXs is still undefined and may have to do with the extent of multiplicity and induction. I have tried to extract from the literature a comprehensive understanding of CTXs which there are still gaps in our knowledge. Metcalf (1975) may be appropriate. No matter how enthusiastic your observations, if your paper is re-read in 10 years the data will almost certainly be much less than 10 years in the

our knowledge of CTXs concerning IFNs. For a survey of other aspects

of CTX research there are a number of useful reviews (Rosenau, 1980; Klostergaard *et al.*, 1981; Ruff and Gifford, 1981a; Ruddle *et al.*, 1983).

## II THE CYTOTOXINS

### A Terminology

There is at present some ambiguity in the terminology of the CTXs. Terms coined in the past for crude preparations of CTX have now been adopted for the purified proteins (Table I). Yet cytotoxic activities of the crude preparations cannot always be fully accounted for by those of the isolated proteins bearing the same name. Furthermore, we now know that there is a significant overlap in the kinds of CTXs present in crude preparations that were given differing names. To avoid misunderstanding, I have chosen to refer in this review to all crude preparations of the proteins by the general term cytotoxins, specifying when necessary the conditions of induction. (An exception is the term 'NKCF' which will be used here, as elsewhere, for describing the CTXs produced by NK cells in response to tumor cells.) The terms 'lymphotoxin' and 'TNF' will be applied solely in reference to the purified proteins.

Nevertheless, it may be useful to review briefly the various terms for the crude CTX preparations formerly in use, as a way of introducing some of the major milestones in CTX research. The term 'lymphotoxin' (LT) was coined for CTX preparations induced in lymphocyte cultures by mitogenic lectins and by antigens. These were first described by Granger and Kolb (1968) and by Ruddle and Waksman (1968). Later studies, primarily by G. Granger and his collaborators, demonstrated heterogeneity in physicochemical as well as in serological properties of PHA-induced CTXs. Differing fractions of such preparations, separated by gel filtration and by ion-exchange chromatography, have been termed 'LT- $\alpha$ ,  $\beta$  and  $\gamma$ ' for what appeared to be main size populations of the CTXs. LT- $\alpha$ 1 denoted a subfraction of LT- $\alpha$  in DEAE-cellulose chromatography, and so on (*cf.* Granger *et al.*, 1978).

Production of some cytotoxic factors also by cultured mononuclear phagocytes (MP) was noted quite some time ago (*cf.* Pincus, 1967). However, the first detection of that specific CTX we now call 'tumour necrosis factor' (TNF) can be traced to a study in 1975, describing induction of haemorrhagic necrosis in a transplantable methylcholanthrene-induced sarcoma by *in vivo* produced CTXs. These CTXs were induced by injecting mice with bacterial lipopolysaccharides (LPS) following priming with Bacillus-Calmette Guérin (Carswell *et al.*, 1975). Initially circumstantial evidence, and later serological

TABLE I Modes of Induction, Terms Used for Differing Preparations of CTXs and Identity of Concomitantly Formed IFNs

Producer cells	Inducing agents	Terms for crude preparations of induced CTXs	Terms for isolated CTXs	IFNs induced
T-cells (primarily Lyt-1 <sup>+</sup> )	T-cell mitogens (antigens; lectins; allogeneic cells; IL-2 . . .)	Lymphotoxins (LTs)	LT (or TNF- $\beta$ ) (and TNF- $\alpha$ ?)	IFN- $\gamma$
Lymphoblastoid B cells	4 $\beta$ -phorbol-12-myristate- 13-acetate (PMA)			
Mononuclear phagocytes	LPS; PMA; Sendai virus; Tumour cells.	Tumour necrosis factor (TNF)	TNF (or "Necrosin" or TNF- $\alpha$ or "cachectin")	IFN- $\alpha$ and $\beta$
NK cells	Tumour cells	Natural killer cytotoxic factor (NKCF)	(TNF ?)	IFN- $\alpha$

examination (Männel *et al.*, 1980), affirmed the identity of these CTXs produced *in vivo* with those produced *in vitro* by cultured mononuclear phagocytes (MP).

More recent studies have demonstrated production of CTXs also by natural-killer (NK) cells, in response to certain tumour target cells; those CTX preparations were termed 'natural killer cytotoxic factors' (NKCF) (Wright and Bonavida, 1982).

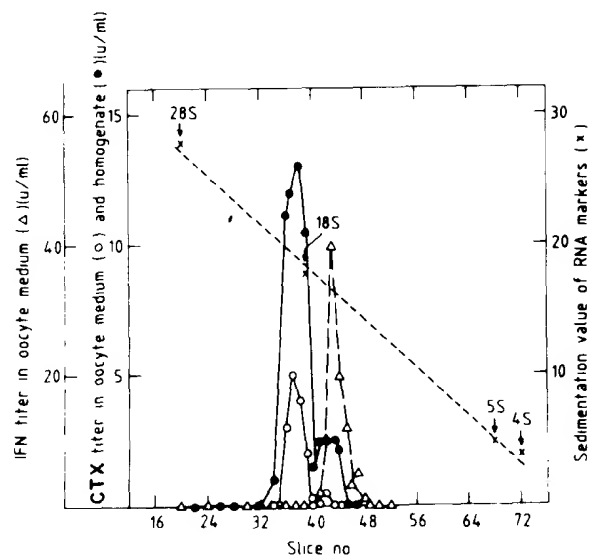
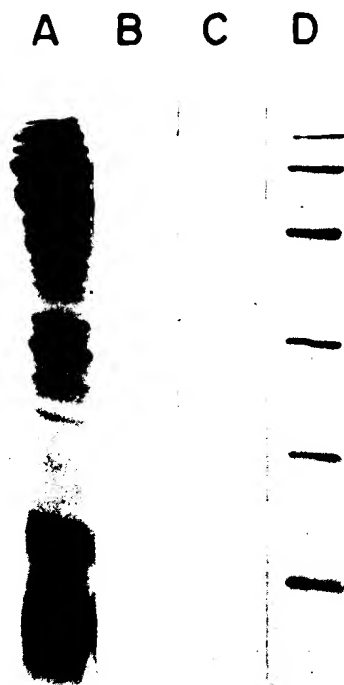
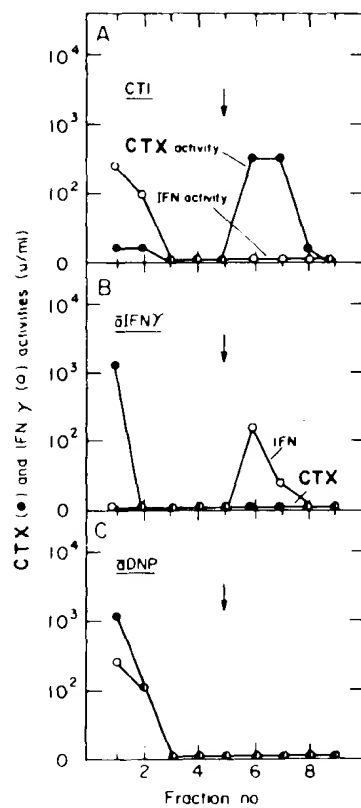
#### B Characteristics of the Isolated CTXs.

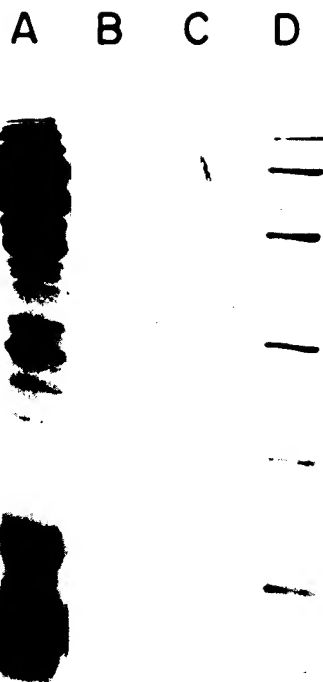
Crude preparations of lymphotoxins, tumour necrosis factor and NKCF all contain IFNs. Since IFNs by themselves can exert cytostatic and cytotoxic activities, separating the two kinds of factors depends on first defining functional criteria whereby they differ from each other. The most evident difference between the two is that CTXs do not have the antiviral activity that characterizes the IFNs. In addition, clear differences can also be discerned in the way IFNs and CTXs exert their anticellular effects: killing of cells by IFNs, like all other effects of the IFNs, is dependent on synthesis of some proteins in the affected cell. On the other hand, cell killing by CTXs is found to be mediated independently of protein synthesis and, in fact, to be greatly potentiated by inhibitors of protein and of RNA synthesis. Furthermore, species-specificity to the extent observed in IFN function is not observed in the cytotoxic function of CTX preparations.

As shown in Fig. 1, which demonstrates separation of a CTX (TNF) from an IFN (IFN- $\gamma$ ) and separation of the mRNAs for the two proteins, these functional differences between CTXs and IFNs can allow specific detection of the one in the presence of the other. IFNs can be determined in the presence of CTXs by measuring the antiviral effect of the former, while CTXs can be specifically determined in the presence of IFN by measuring cytotoxic effects exerted in the presence of inhibitors of RNA or of protein synthesis. Alternatively, the cytotoxic activity of CTXs can be specifically determined in the presence of IFNs by using cells of a remote animal species which would not respond to the species-specific IFN effect (e.g. use of mouse L929 cells for detecting human TNF).

Two CTXs have recently been isolated—one, a glycoprotein with an  $M_r$  of about 25 Kd was isolated from preparations of CTXs produced by cells of a B-lymphoblastoid cell line (Aggarwal *et al.*, 1984) and the other, a non-glycosylated protein, with an  $M_r$  of about 17 Kd—from preparations of the CTXs induced in cells of a promyelocytic cell line (Aggarwal *et al.*, 1985a; Haranaka *et al.*, 1985). The two proteins turned out to be closely related in structure, so that while initially they were called lymphotoxin (LT) and tumour necrosis factor (TNF), it was suggested recently that they be named

Lymphoblastoid B cells	4 $\beta$ -phorbol-12-myristate-13-acetate (PMA)	Tumour necrosis factor (TNF)	TNF (or "Necrosis" or TNF- $\alpha$ or "cachectin") (TNF ?)	IFN- $\alpha$ and $\beta$
Mononuclear phagocytes	LPS; PMA; Sendai virus; Tumour cells.	Natural killer cytotoxic factor (NKCF)		IFN- $\alpha$
NK cells	Tumour cells			





TNF  $\beta$  and  $\alpha$  respectively. The mouse homologue of TNF was initially called 'necrosin' (Kull and Cuatrecasas, 1984). This term was coined by Menkin (1943), for an *in vivo*, induced factor, recovered from pleural exudates of dogs injected with turpentine, that could induce cellular injury in animals resembling that observed in inflammation.

The amino acid sequence of LT and TNF, as established from analysis of the proteins themselves as well as from sequencing their complementary and genomic DNAs, were found to be remarkably homologous (Aggarwal *et al.*, 1984, 1985; Gray *et al.*, 1984; Pennica *et al.*, 1984; Shirai *et al.*, 1985; Wang *et al.*, 1985; Fransen *et al.*, 1985). By introducing gaps in the structure of LT, the two sequences can be aligned with an identity of 28% and with homology (including conservative changes) of 46% of their amino acids. Differences are largely confined to a single region in the molecules (residues 69–101 in TNF and 86–118 in LT). That 32-amino-acid region is, in TNF, confined by a disulfide bridge (between cysteins 69 and 101) which probably imposes on this sequence a loop structure separated from the rest of the molecule.

The similarity in structure between TNF and LT is reflected in their functions which, so far, appear to be indistinguishable. However, the differences in structure between the two proteins may yet be found to be functionally meaningful. The sequence homology between TNF and LT is significantly less than that between human and mouse TNF (Fransen *et al.*, 1985; Marmenout *et al.*, 1985; Pennica *et al.*, 1985). Almost complete homology (including conservative changes) in the amino acid sequences of TNF from the two sources is observed not only throughout the mature molecules but also in the structure of their leader portion, which in TNF is significantly longer (76 residues) than in LT (34 residues): it is encoded by exons distinct from those coding for most of the mature protein. The effective preservation,

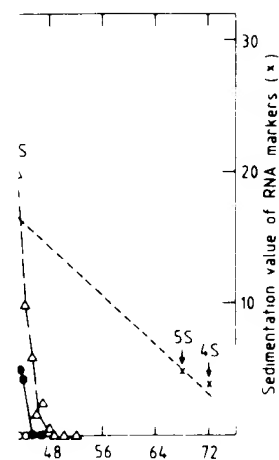
Fig. 1. (A) and (B) Isolation of TNF by the use of a monoclonal antibody (mAb) to the protein (Hahn *et al.*, 1985). (C) Dissociation of the mRNAs for TNF and IFN- $\gamma$  using the oocyte microinjection technique for determining translational activity of the two mRNAs (Wallach *et al.*, 1984).

(A) Fractionation of a cytokine preparation containing the two proteins using immuno-adsorbents constructed with mAbs against TNF (A), IFN- $\gamma$  (B) or dinitrophenyl (C). Bound proteins were eluted with 0.2 M NH<sub>4</sub>OH (arrow).

(B) SDS, 15% PAGE of TNF purified by immunoadsorption. The crude preparation of the protein (A); NH<sub>4</sub>OH-eluted fraction from the immuno-adsorbent constructed with the anti-dinitrophenyl mAb (B); TNF purified on the mAb against this protein (C); molecular weight standards (94; 67; 43; 30; 20.1 and 14.4) (D).

(C) 1.2% agarose-methylmercuric hydroxide gel electrophoresis of poly(A)<sup>+</sup> RNA from stimulated human PBMC. RNA extracted from slices of the gel was microinjected into *Xenopus* oocytes and the level of cytotoxic activity in the oocyte homogenates (●) as well as the cytotoxic (○) and IFN activity (Δ) in the oocyte incubation media were determined 24 hours following microinjection.

TNF activity was determined by measuring cytotoxic effect on SV-80 cells in the presence of CHI and IFN-activity by determining reduction in the cytopathic effect of Vesicular stomatitis virus (VSV) on WISH cells.



through evolution, of parts of TNF differing from LT implies that these sequences are functionally important and that their alteration in the evolution of LT may be functionally meaningful.

At present it is not clear how many other CTXs there are besides TNF and LT. The recently described 'perphorins' are CTXs that appear to function quite differently from LT and TNF and are therefore likely to differ from these proteins in structure as well. The perphorins are components of cytoplasmic granules of cytotoxic T-lymphocytes and of NK cells. Upon interaction of the isolated granules or of the intact cytotoxic cells with membranes the proteins form, in a  $\text{Ca}^{2+}$ -dependent manner, tubular complexes that are inserted into the membranes and cause cytolysis (Podack (1985)). Purification of "perphorin" and initial characterization of its molecular properties have recently been reported (Young *et al.*, 1986).

There have been a few reports of purification of some other CTXs that apparently differ from TNF and LT (e.g. Ransom *et al.*, 1985; Rubin *et al.*, 1985). Moreover, some proteins of non leukocyte origin which exert cytostatic effects have also been isolated (e.g. cf. Roberts *et al.*, 1985). It is not yet known to what extent, if any, these proteins are structurally related to TNF and LT.

### III CTX FORMATION AND ITS ENHANCEMENT BY IFN

#### A Production of CTXs and of IFN in Response to the Same Inducing Agents

A considerable number of agents were found to induce the production of CTXs. As demonstrated in Fig. 2, effective production of CTXs can be induced in human peripheral blood mononuclear cells (PBMC) by agents as different from each other as phytohaemagglutinin (PHA) and Sendai virus. These two agents induce the proteins by affecting different kinds of leukocytes. Sendai virus induces the production of CTXs in MP, while PHA induces CTXs by stimulating non-adherent mononuclear cells (apparently T-cells) (Table II). Other T cell mitogens—such as foreign antigens—also induce CTXs in T cells (Ruddle *et al.*, 1985). In addition, bacterial lipopolysaccharides induce CTXs in MP (Ruff and Glifford, 1981a); the diterpene tumour promotor 4- $\beta$ -phorbol-12-myristate-13-acetate (PMA) induces CTXs in MP as well as in certain lymphoid B and T cell lines (Gifford *et al.*, 1984; Williamson *et al.*, 1983; Adolf, 1984) and tumour cells can induce CTXs both in MP and in NK cells (Männel, 1981; Wright and Bonavida, 1982). The main CTX found to be produced by MP is TNF, while lymphocytes were reported to produce primarily LT (Chroboczek-Kelker *et al.*, 1985).



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differing from LT implies that these and that their alteration in the evolution.

her CTXs there are besides TNF and are CTXs that appear to function and are therefore likely to differ from perphorins are components of cyto-cytes and of NK cells. Upon interac-tact cytotoxic cells with membranes manner, tubular complexes that are e cytotoxic (Podack (1985)). Purifi-cation of its molecular properties *et al.*, 1986).

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## ENHANCEMENT BY IFN

Response to the Same Inducing

ound to induce the production of active production of CTXs can be monuclear cells (PBMC) by agents as agglutinin (PHA) and Sendai virus. affecting different kinds of leuko-cytes in MP, while PHA ent mononuclear cells (apparently s—such as foreign antigens—also 5). In addition, bacterial lipopoly-saccharide (LPS) (Gifford, 1981a); the diterpene state-13-acetate (PMA) induces phorbol B and T cell lines (Gifford and Jolff, 1984) and tumour cells can be produced by MP is TNF, to be produced by MP is TNF, duce primarily LT (Chroboczek-

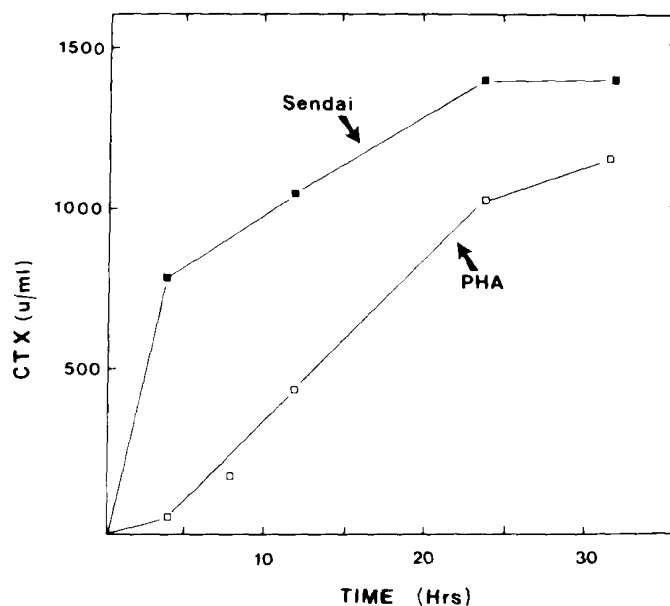


Fig. 2. Induction of CTXs in human PBMC ( $10^7$  cells/ml) with Sendai virus (500 HA/ml) and with PHA ( $5 \mu\text{g/ml}$ ) (Aderka *et al.*, 1986a).

TABLE II Differing Responsiveness of Mononuclear Phagocytes and of the Nonadherent Fraction of PBMC to Different Inducers of CTXs

Inducer	CTX Yields (U/ml)	
	MP	Nonadherent PBMC (Primarily T-lymphocytes)
None	10	< 5
PHA ( $5 \mu\text{g/ml}$ )	15	95
Con-A ( $20 \mu\text{g/ml}$ )	15	35
LPS ( $10 \mu\text{g/ml}$ )	30	< 5
PMA ( $5 \text{ ng/ml}$ )	330	10
Sendai virus (500 HA/ml)	3000	15
LPS + PMA	2600	10

CTX induction (for 24 hours) by various agents, in fractionated human peripheral-blood mononuclear cells (PBMC) (Aderka *et al.*, 1986a).

Agents that stimulate T lymphocytes may, however, be found to induce significant production of TNF as well in cultures of PBMC, apparently through indirect stimulation of MP by the stimulated T cells (Aderka *et al.*, 1986a; Nedwin *et al.*, 1985). The identity of the CTXs comprising NKCF preparations is not yet clear. There have been preliminary reports suggesting that these preparations may contain TNF as well as CTXs that differ from TNF (Svedersky *et al.*, 1985a; Ortaldo *et al.*, 1985).

Those agents that induce production of CTXs in the various leukocytes also induce IFNs in the same cells (Table I): T cell mitogens induce in T cells the production of IFN- $\gamma$  together with LT. Agents that stimulate MP to produce TNF also induce in these cells IFN- $\alpha$  and IFN- $\beta$ , while in NK cells tumour cells induce the production of IFN- $\alpha$  as well as NKCF. Indeed, significant amounts of IFN would be detected in preparations of CTXs (see Matthews 1979; Ware and Granger, 1979).

#### B Enhancement of CTX Production by IFN

To find out if the IFNs produced with CTXs have a regulatory role in CTX induction, we have compared the efficiency of CTX production by PBMC pretreated with IFN- $\beta$  to CTX production by untreated cells. No CTXs were induced as a result of treatment by IFN. However, when challenged by PHA, the IFN-treated cells produced CTXs at a significantly higher rate than untreated cells (Wallach and Hahn, 1983) (Fig. 4A). Furthermore, it could be shown, using antibodies to IFN, that production of CTXs by PHA-stimulated leukocytes that had not been pretreated by IFN was augmented, during prolonged incubation, by the IFN- $\gamma$  produced *in situ* (Wallach *et al.*, 1983).

Enhancement of CTX production by IFN has turned out to be the rule for a variety of processes in which CTXs are formed, including the induction of CTXs in PBMC by antibody-coated cells IL-2, Concanavalin A (Con A), or staphylococcal enterotoxin A where, with the use of specific antibodies to TNF and LT it could be shown that production of both these CTXs were enhanced (Wallach *et al.*, 1983; Svedersky *et al.*, 1985b; Nedwin *et al.*, 1985). Production of TNF by mononuclear phagocytes in response to LPS and of NKCF by NK cells in response to tumour target cells was also found to be enhanced by IFN (Kildahl-Anderson *et al.*, 1985; Farram and Targan, 1983; Wright and Bonavida, 1983a; Steinhauer *et al.*, 1985). Furthermore, injection of IFN- $\gamma$  into mice potentiated the production of TNF in subsequent injection of LPS (G. E. Gifford, and M. L. Lohmann-Matthes, personal communication). Augmentation *in vivo* of TNF production by IFN in man could be observed in patients with hairy-cell leukemia. The PBMC of these patients produce TNF poorly in response to stimulation *in vitro* by PHA, PMA or

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stimulated T cells (Aderka *et al.*,  
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Sendai virus. However, following a month of treatment by IFN- $\alpha$ , produc-  
tion of TNF in response to PHA increases, reaching close to normal values.  
This augmentation appears to reflect both increase in counts of MP  
and in responsiveness of the cells to induction of TNF (Aderka *et al.*,  
1986b).

## C Mechanisms Involved

There is inherent complexity in the processes of CTX induction and in their  
regulation. Within the CTX-producing cell, components and mechanisms at  
differing subcellular sites take part in the sequence of events that lead from  
the initial interaction of the cell with a CTX inducer to the eventual secretion  
of the CTXs. Furthermore, indirectly, mechanisms functioning outside the  
CTX-producing cell may also affect the formation of CTXs. Since CTX  
formation may be induced by cell-associated components (e.g. foreign anti-  
gens on the surface of cells), whose expression can be modulated, mechanisms  
functioning within such CTX-inducing cells may have a regulatory role in the  
production of CTXs. In addition, as discussed below, the production of  
CTXs may be affected by regulatory cytokines, whose own formation can be  
modulated by regulatory agents. There are thus multiple potential sites for  
regulation by IFN of the processes involved in CTX induction.

Direct information on mechanisms that participate in CTX production is  
scarce. However, based on indirect information I list below cellular activities  
and components likely to take part in the processes of CTX induction, in  
order to point out effects of IFN through which these processes can be  
modulated. These putative sites for regulation of CTX production by IFN,  
as well as the mode of regulation by IFN of CTX effects on cells, are depicted  
diagrammatically in Fig. 3.

## 1 Regulation of CTX formation at the level of CTX-producing cells

Since CTX-inducing agents are primarily extracellular, while the production  
of CTXs occurs within the cell, there are likely to be cell surface receptors or  
sensing mechanisms for these agents that initiate the production of CTXs.  
Such sensors are the T cell antigen receptors, which are probably involved in  
the induction of CTXs in T cells by antigens; the Fc receptor, which is likely  
to take part in induction of CTXs as part of antibody-dependent cytotoxicity  
(ADCC); membrane structures with which LPS interacts when inducing  
CTXs in MP; and others. We know very little about the nature of the  
intracellular mediating mechanisms that transduce the signal for CTX induc-  
tion. Cyclic AMP, a mediator in many hormonal processes, is unlikely to be  
involved, at least not as a positive signal, since agents that increase cyclic

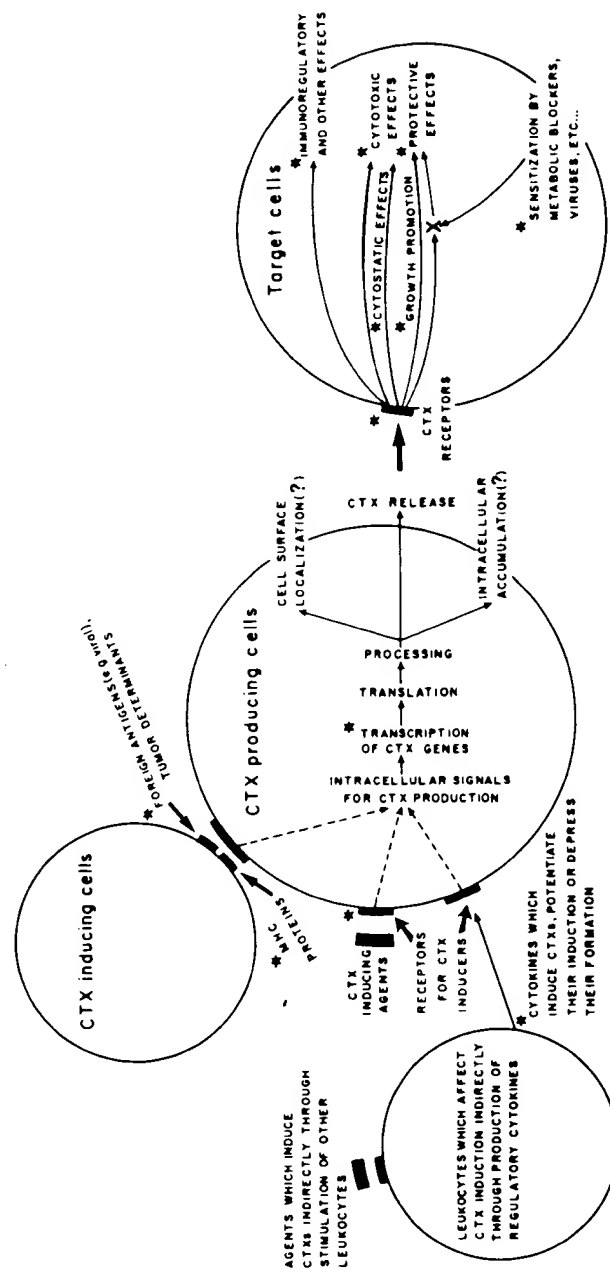


Fig. 3 Putative points of regulation by IFN (denoted by asterisks) in processes of CTX formation and in the response of target cells to the effect of CTXs.

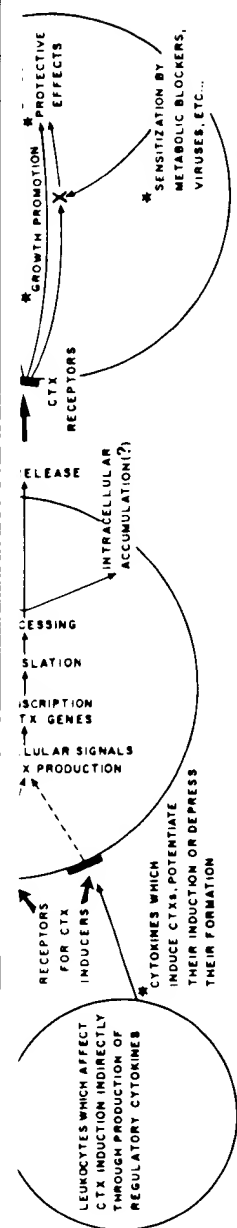


Fig. 3. Putative points of regulation by IFN (denoted by asterisks) in processes of CTL formation and in the response of target cells to the effect of CTXs.

AMP level in leukocytes have been found to suppress the formation of CTXs (Wallach *et al.*, 1983).

One may speculate, on the basis of effects of calcium modulating agents on the production of CTXs by lymphocytes, and on the basis of the ability of the diterpene ester PMA to induce CTXs in MP and in lymphocytes (Wallach *et al.*, 1983; Williamson *et al.*, 1983; Gifford *et al.*, 1984 and Adolf, 1984), that calcium fluxes or protein phosphorylation initiated by protein kinase C (known to be activated by PMA) play a role as mediating signals in the production of CTXs by these cells.

So far, the only intracellular event in CTX formation monitored with the use of a molecular probe is the transcription of CTX genes. mRNAs for TNF and LT could be detected in CTX-producing cells by hybridization to a cDNA probe (Gray *et al.*, 1984 and Svedersky *et al.*, 1985b) as well as by injection of the RNA into *Xenopus* oocytes, where it could be translated to biologically active TNF molecules (Wallach *et al.*, 1984, and Fig. 1C). With either of these techniques, significant increases in CTX mRNA upon CTX induction could be detected, indicating regulation of CTX formation on the level of synthesis, processing or degradation of mRNA (Wallach *et al.*, 1984 and Svedersky *et al.*, 1985b).

Little information is available as to the nature of post-translational events in CTX induction. In the formation of TNF, post-translational processing, involving the removal of a long signal peptide, has to occur prior to release of these molecules from the cell. The site and mechanism of this processing have not been identified as yet. Indirect evidence based on the effects of protein synthesis and cytoskeletal inhibitors on the release of CTXs by lectin-stimulated lymphocytes, and localization studies using antibodies raised against partially purified preparations of these CTXs, were interpreted as suggesting that CTXs can accumulate in lymphocytes both intracellularly and on the cell surface (see Hiserodt *et al.*, 1977, 1979).

Evidence for intracellular accumulation of NKCF in NK cells has also been presented based on detection of NKCF-like activity in homogenates of these cells (Wright and Bonavida, 1983a). The presence of NKCF in intracellular stores is also indicated by the ability of antibodies to preparations of the large granules of NK cells to block the cytotoxic activity of NKCF (Ortaldo *et al.*, 1985). Furthermore, antibodies raised against partially purified MP-produced CTXs were shown to interact with intracellular proteins in mononuclear phagocytes (Kildahl-Andersen *et al.*, 1985). The use of monospecific antibodies to CTXs should now generate more reliable and detailed information on the subcellular localization of CTXs within CTX-producing cells and on the mechanisms of their release.

In an attempt to clarify the IFN-regulated mechanisms that take part in the enhancement of CTX formation by lectin-stimulated PBMC pretreated

with IFN, we first questioned whether the IFN-induced changes in this case occur in the CTX-producing cells themselves or are the result of a secondary effect, due to altered production of regulatory cytokines secreted by some leukocytes within the heterogeneous population of the PBMC. Preliminary data (Fig. 4) suggest that lectin-stimulated PBMC secrete cytokines, distinct from IFN (or CTXs), that enhance CTX formation as effectively as IFN. When applied on PBMC, IFN-free preparations of these cytokines did not induce production of CTXs (except at significantly higher concentrations than used in our experiments). However, on subsequent stimulation by PHA, PBMC treated at the lower concentrations of cytokines responded much more rapidly than non-treated cells in release of CTXs. In fact, this occurred just as rapidly as CTX release from IFN-pretreated cells (Fig. 4A).

The amounts of cytokines responsible for this effect could be estimated indirectly by determining how effectively the cytokine preparations enhanced CTX formation (Fig. 4B). Thus, it was shown that IFN-pretreated leukocytes produce more of these cytokines than cells that were not treated by IFN (Fig. 4C). Although the cytokines responsible for this effect have not yet been identified, the findings in this experimental system indicate that IFN-induced enhancement of CTX formation, at least in part, indirectly reflects enhanced formation of cytokines which themselves have a stimulatory effect on CTX formation. This complex mode of regulation by IFN may result in enhanced, cascade-like, stimulatory effect.

• CTX formation and function may also be modulated by suppressive factors. The delayed type hypersensitivity (DTH) response, which probably involves CTX formation (see below), can be effectively blocked by suppressor cells. The decreased activity of suppressor cells, observed following treatment by IFN (Knop *et al.*, 1982) may further contribute to an increase in formation of CTXs.

Regulatory cytokines may function also as direct inducers of CTXs. Interleukin-2 was reported to induce effective production of both TNF and LT in cultures of PBMC (Svedersky *et al.*, 1985b; Nedwin *et al.*, 1985). IFN may increase the formation of interleukin-2 by enhancing the production of interleukin-1 and thus indirectly potentiate CTX production.

Modulation of production of regulatory cytokines is likely to contribute to a significant extent to the effect of IFN on formation of CTXs in cultures of mixed populations of leukocytes, where the differing kinds of mononuclear leukocytes indeed have marked regulatory effects on each other through the action of cytokines they secrete. To find out whether direct effects of IFN on CTX-producing cells may also contribute to the enhancement of CTX formation, we have sought to examine this regulation in a simpler experimental system—a homogeneous population of CTX-producing cells of cultured lines. Preliminary results from a study on the effect of IFN on the production

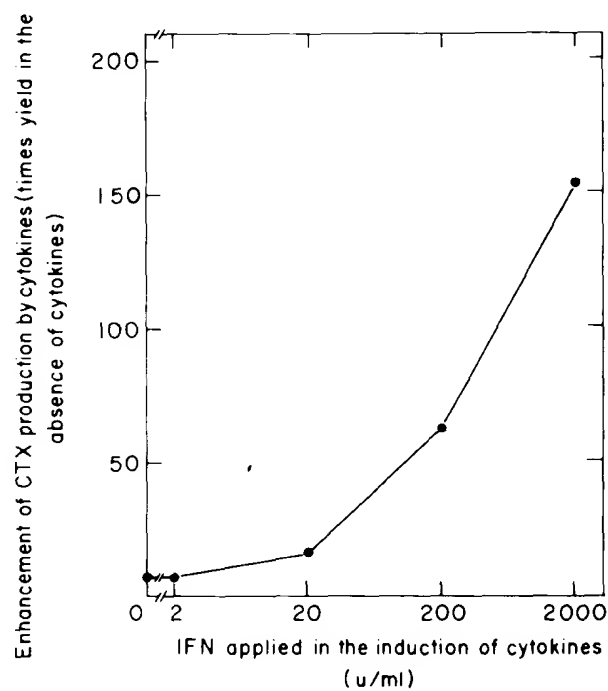
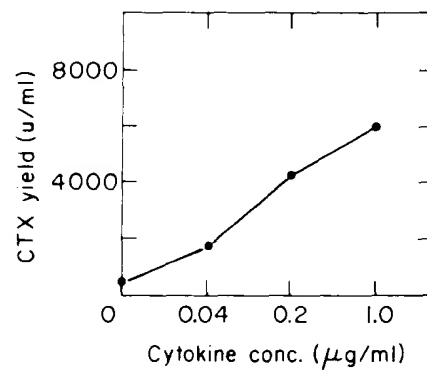
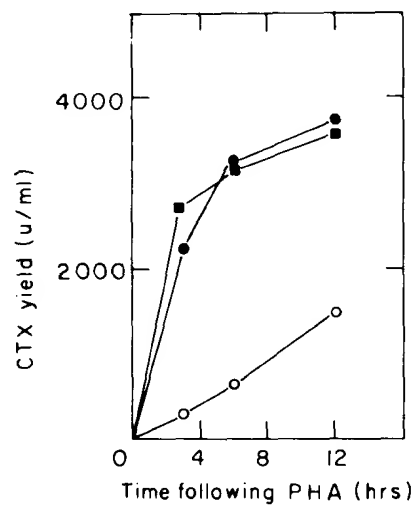
IFN-induced changes in this case or are the result of a secondary cytokines secreted by some of the PBMC. Preliminary PBMC secrete cytokines, distinct from IFN, as effectively as IFN. Concentrations of these cytokines did not differ significantly from higher concentrations of IFN. A subsequent stimulation by PHA, a mixture of cytokines responded much like IFN. In fact, this occurred with pretreated cells (Fig. 4A). The effect of this could be estimated by cytokine preparations enhanced by IFN. IFN-pretreated leukocytes and cells that were not treated by IFN were able for this effect have not yet been tested. The system indicate that IFN-induced in part, indirectly reflects enhanced effect have a stimulatory effect on CTX production by IFN may result in enhanced, so be modulated by suppressive (DTH) response, which probably is effectively blocked by suppressor cells, observed following treatment contribute to an increase in formation of CTXs. Also as direct inducers of CTXs. Active production of both TNF and IFN (1985b; Nedwin *et al.*, 1985). IFN- $\gamma$  by enhancing the production of CTX production. Cytokines is likely to contribute to formation of CTXs in cultures of cells differing kinds of mononuclear cells. The effects on each other through the direct effects of IFN on the enhancement of CTX formation in a simpler experimental CTX-producing cells of cultured cells. The effect of IFN on the production

TABLE III Increase in Production of CTXs by U937 Cells Following Treatment by IFN, TNF or a Crude Leukocyte-produced Cytokine Preparation.

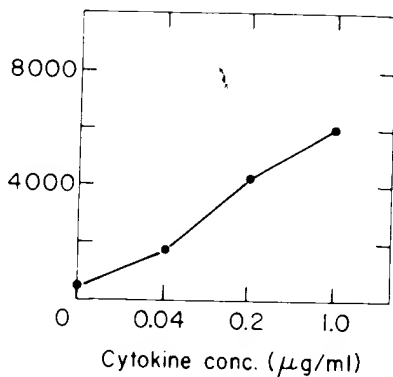
Preincubation	CTX yield (U/ml)		
	no inducing agents	LPS	Sendai virus
-	< 5	< 5	< 5
PMA	10	180	125
rIFN- $\gamma$	< 5	< 5	< 5
PMA + IFN- $\gamma$	35	500	625
PMA + TNF	10	440	N.T.*
PMA + TNF + IFN- $\gamma$	40	1030	N.T.
PMA + crude leukocyte cytokines	N.T.	3130	N.T.

U937 cells ( $10^6$  cells/ml) in samples of 2 ml were incubated in 18 mm tissue culture wells for 24 hours with the agents indicated in the first column, then rinsed, brought to 1 ml and incubated further for 24 hours either alone or with LPS or with Sendai virus. Yields of CTXs in the cultures were then determined by measuring cytotoxic effect on CHI sensitized L132 cells. PMA was applied at 5 ng/ml, rIFN- $\gamma$  at 1000 U/ml, TNF (native; homogeneously purified on the CT-1 mAb) at 100 U/ml, crude leukocyte cytokines (see below) at 0.2  $\mu$ g/ml, LPS at 10  $\mu$ g/ml and Sendai virus at 500 HA/ml. Leukocyte cytokines were induced in PBMC by 36 hours' stimulation with PMA (5 ng/ml) and Con-A (20  $\mu$ g/ml), then concentrated by adsorption to controlled-pore glass and treated at pH 2.0 as described in the legend to Fig. 4. No IFN or CTX activity could be detected in this kind of preparation (unpublished observations).  
\*N.T.: not tested.

of CTXs by the human histiocytic lymphoma U937 cells are presented in Table III. Although unable to produce CTXs under normal growth conditions, the U937 cells do produce CTXs (primarily TNF) when induced to differentiate with PMA, and produce CTXs to an even greater extent when further treated either with LPS or with Sendai virus. As shown in Table III, IFN- $\gamma$  enhances formation of the CTXs by the U937 cells. Although even in this case an indirect effect of IFN through induction of "autokines", which the U937 cells both produce and respond to, cannot be ruled out, it seems more likely that IFN enhances the production of CTXs in the U937 cells by inducing changes in cellular mechanisms directly involved in the process of CTX formation. Effects of regulatory cytokines other than IFN on CTX production may also be studied in the U937 cells. TNF itself potentiates the production of CTXs (actually its own production) in these cells, and that potentiation effect is additive to the effect of IFN- $\gamma$ . Crude preparations of leukocyte-produced cytokines, free of both IFN and TNF, also have very strong potentiation effects on the production of CTXs by those cells (Table III).  
Among the various components and mechanisms within the CTX-producing cell whose alteration by IFN may result in augmented production of the CTXs, one may first point out two specific receptors involved in these



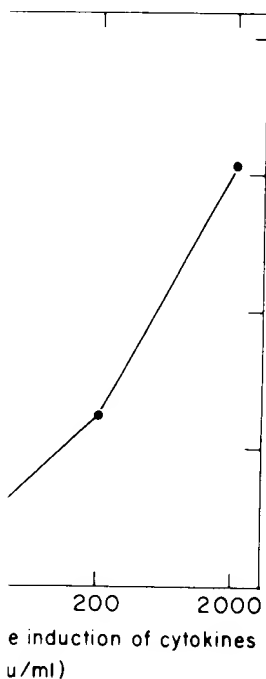




processes: the Fc receptor, whose increase by IFN may contribute to the augmented production of CTXs in antibody-dependent cytotoxic reactions, and the IL-2 receptor, which is also increased by IFN and which is likely to be involved in the induction of CTXs by T cell mitogens as well as by IL-2 itself. Recent findings by Sverdersky *et al.* (1985b) are consistent with the idea that transcription of mRNAs for CTXs is also enhanced by IFN. The level of mRNA for this CTX, in PBMC stimulated by IL-2 to produce CTXs, was estimated with the use of a cDNA probe for LT. In non-stimulated PBMC the concentration of mRNA for LT was below the level of detection; however, following treatment by IL-2, significant accumulation of the mRNA could be observed. In correlation with the effect of IFN on the production of LT itself, the mRNA for this protein did not increase on treatment by IFN- $\gamma$  alone; but IFN treatment did enhance significantly the increase in mRNA observed on subsequent treatment with IL-2.

Fig. 4 IFN-induced increases in the production of CTXs and of cytokines which enhance CTX formation. (A) Kinetics of CTX production by PBMC in response to stimulation by PHA following pretreatment by IFN- $\alpha$  (1000 U/ml) (●) or by IFN-free leukocyte-produced crude cytokine preparation (0.2  $\mu$ g/ml) (■), compared to CTX production in PBMC not pretreated by IFN nor by cytokines (○). (B) Titration of the stimulatory effect of the cytokine preparation on production of CTXs by PBMC in response to 6 hours' stimulation with PHA. (C) IFN-induced increase in production of the cytokines which enhance CTX formation.

PBMC isolated from freshly donated blood on a Ficoll-Hypaque cushion, were suspended in MEM-alpha medium at a concentration of  $10^7$  cells/ml, incubated for 12 hours with IFN or with the cytokines and then rinsed and treated with PHA-P (5  $\mu$ g/ml). Cytotoxic activity in the culture medium was determined with the use of CHI-sensitized SV80 cells. The cytokine preparations were produced by treating PBMC for 12 hours with IFN- $\alpha$  (2000 U/ml in A, B and at varying concentrations in C) then rinsing the cells 3 times and applying Concanavalin A (Con-A 20  $\mu$ g/ml) for 24 hours (6 hours in C). In A and B these cytokine preparations were concentrated by adsorption to controlled pore glass, eluted with 0.5 M tetramethylammonium chloride, further concentrated by ultrafiltration and incubated for 12 hours at pH 2.0. The ConA was eliminated in the adsorption to controlled pore glass. Acidification resulted in complete loss of IFN and of CTX activity of the cytokine preparation with no decrease in the effectiveness by which the cytokines enhanced CTX formation. Depletion of IFN- $\gamma$  by applying these cytokine preparations on an immunoadsorbent constructed from a monoclonal antibody against that IFN did not decrease the effectiveness by which those cytokines augmented production of CTXs either. Inclusion of 50 mM  $\alpha$ -methyl-D-mannopyranoside during CTX induction, to interfere with the function of any residual Con A, did not decrease the enhancement of CTX formation. Protein concentration in the crude cytokine preparation was about 1  $\mu$ g/ml of leukocyte culture. In C, the preparations of cytokines (produced by cells pretreated with various concentrations of IFN- $\alpha$  and then stimulated for 6 hours with Con A) were not concentrated on controlled pore glass but just brought to pH 2.0 and 12 hours later returned to pH 7.0. The preparations were then applied at a dilution of 1:5 on PBMC in the presence of  $\alpha$ -methyl-D-mannopyranoside (50 mM). The cells were incubated with the lymphokines for 12 hours and, after rinsing, for 6 further hours with PHA-P (5  $\mu$ g/ml), yields of CTXs were determined. Yields of CTXs in the absence of pretreatment by cytokines were 68 U/ml in B and 11 U/ml in C. Electrophoretically pure native IFN- $\alpha$  was used in all experiments (unpublished observations).



## 2 Regulation at the level of CTX inducing cells

Additional potential sites for regulation by IFN can be discerned in those processes for CTX production in which the inducing agents are cells—cells which present foreign antigens on their surface and which induce CTXs in T lymphocytes, and tumour cells that induce CTXs in NK cells and in mononuclear phagocytes. The role of IFN in the production of CTXs by antigen-stimulated T cells has not, to my knowledge, yet been examined. In theory though, we may expect, that IFN will effectively modulate these processes of CTX production by inducing two kinds of changes: increase in major histocompatibility (MHC) proteins, and the antiviral effect, neither when induced in the CTX-producing T cells but in the cells that present the stimulating antigens to the T-cells.

As in other processes of antigen-induced T cell activation, in the induction of CTXs in T cells, antigens have to be presented in association with the MHC proteins. This refers not only to cell-associated foreign antigens, but also to soluble antigens, since these are recognized by T cells only after being processed (primarily by MP) and exposed on the surface of the processing cell in association with MHC proteins.

Of the two major classes of the MHC proteins (Class I proteins HLA-A, -B, -C in man and H2-K and -D in mouse, and Class II proteins including HLA-DR in man and Ia in mouse), it seems that the class II proteins primarily take part in CTX induction. After fractionation of T cells, most cells capable of producing CTXs in response to antigens were Lyt-1<sup>+</sup> cells, known to recognize antigens in the context of the class II MHC proteins. Little CTX induction could be detected in the Lyt-2<sup>+</sup> cells, which recognize antigens associated with the class I MHC proteins (Eardley *et al.*, 1980; Tite *et al.*, 1985). IFNs, most notably, IFN- $\gamma$ , enhance synthesis of the MHC proteins, particularly the class II MHC proteins (Lindahl *et al.*, 1974; Wallach, *et al.*, 1982; Basham and Merigan, 1983).

In a variety of cells, synthesis of the class II MHC proteins may, in fact, be observed only upon treatment by IFN- $\gamma$ . IFN may in this way greatly promote antigen-induced CTX production. However, IFN may suppress induction of CTXs by antigens when these are virus-derived. This may not be an exceptional situation, since infecting viruses are probably the most common physiological source of cell-associated foreign antigens. Many viruses, however, do not respond to the antiviral effect; cell surface expression of viral antigen may even be found to be increased by IFN (Chang *et al.*, 1977).

On the whole, the combined effects of IFN on the MHC antigens and on the expression of viral antigens on the cell surface may be expected to result in decreased CTX induction by virus-infected cells whenever IFN suppresses

g cells

by IFN can be discerned in those he inducing agents are cells—cells rface and which induce CTXs in T e CTXs in NK cells and in mono- e production of CTXs by antigen- dge, yet been examined. In theory ctively modulate these processes of f changes: increase in major hysto- tiviral effect, neither when induced cells that present the stimulating

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proteins (Class I proteins HLA-A, se, and Class II proteins including seems that the class II proteins fter fractionation of T cells, most onse to antigens were Lyt-1<sup>+</sup> cells, ext of the class II MHC proteins. n the Lyt-2<sup>+</sup> cells, which recognize proteins (Eardley *et al.*, 1980; Tite  $\gamma$ , enhance synthesis of the MHC proteins (Lindahl *et al.*, 1974; an, 1983).

ass II MHC proteins may, in fact, N- $\gamma$ . IFN may in this way greatly on. However, IFN may suppress se are virus-derived. This may not ng viruses are probably the most sociated foreign antigens. Many tiviral effect; cell surface expression e increased by IFN (Chang *et al.*,

IFN on the MHC antigens and on l surface may be expected to result ted cells whenever IFN suppresses

replication of the virus, and in enhanced induction of CTXs by infected cells that fail to respond to the antiviral effect of IFN.

There is evidence that in the induction of CTXs in NK cells, too, IFN has a regulatory role at the level of the CTX-inducing cells. Mouse Yac cells, which can induce the production of NKCF, become less effective inducers when treated by IFN (Wright and Bonavida, 1983b). That observation is consistent with prior studies showing that IFNs, most notably IFN- $\gamma$ , can suppress NK cytotoxicity by inducing resistance to killing in the target cells (Trinchieri and Santoli, 1978; Wallach, 1983). There is as yet no information on the molecular nature of these IFN-induced changes in NK-target cells or on the nature of the target structures for NK-cells in general.

#### IV CTX EFFECTS AND THEIR POTENTIATION BY IFN

##### A CTX Effects

###### 1 Cytotoxic and cytostatic effects

In most studies on the cytotoxic effect of CTXs, the target cells have been L929 cells, which are particularly sensitive. From the limited characterization of the effect of CTXs on other cells, it appears that there is a wide spectrum of cells that are potentially sensitive to CTXs. However, in most of these cells the cytotoxic effect is rather slow, occurring in incubation for several days. A rapid cytotoxic effect, occurring within a few hours of CTX application, would not be observed unless the cells had been sensitized in some way. Such sensitization can be achieved by treating cells with metabolic blockers such as actinomycin D, cycloheximide (CHI), mitomycin-c, O-dinitrophenyl or sodium azide (Williams and Granger, 1973; Rosenau *et al.*, 1973; Ruff and Gifford, 1981b; Wallach, 1984, unpublished observations). It can also be induced by x-irradiation or by infection of the CTX-treated cells with certain viruses (Eifel *et al.*, 1979; Aderka *et al.*, 1985; and Figs 5 and 6).

Cells of several lines, including some that are quite resistant to the cytotoxicity of CTXs, can be found to respond to CTXs by arrest of cell growth. The relation of this cytostatic effect of CTXs to the cytotoxic activity is not clear. Possibly growth arrest by these proteins is, in many cases, an early reflection of cellular changes that eventually result in cell death. However, at least in some cells, growth arrest by CTXs may reflect specific inhibition of growth-related functions, as indicated by the fact that it results in accumulation of cells in specific stages of the cell cycle. Thus L929 cells were reported to respond to TNF in an arrest at G<sub>2</sub> (Darzynkiewicz *et al.*, 1984), and B16

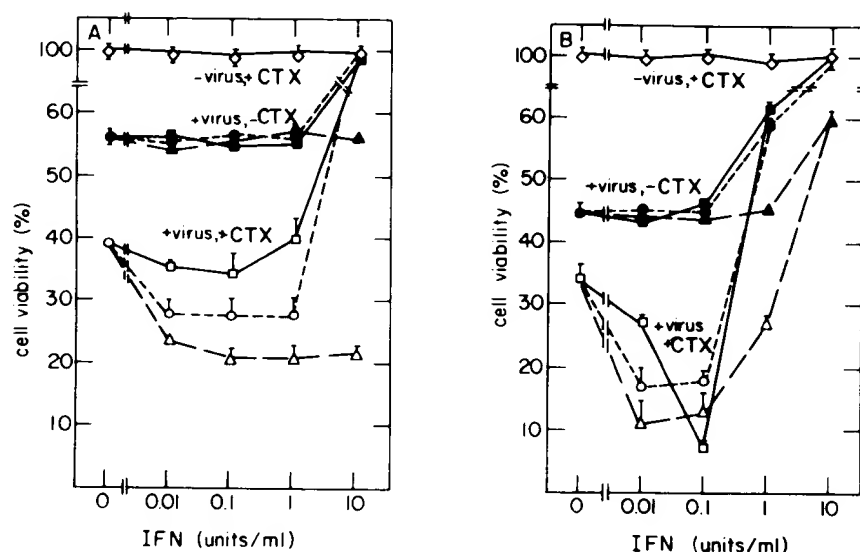
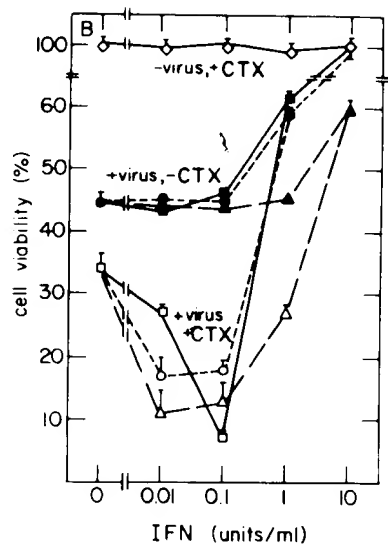


Fig. 5 IFN effects on the cytotoxicity of CTXs in SV80 cells (A) and in HeLa cells (B). IFN- $\alpha$  (○, ●), IFN- $\beta$  (□, ■), and IFN- $\gamma$  (△, ▲) were applied at the indicated concentrations prior to infection by VSV (solid symbols) or infection by VSV followed by application of CTX (6 U/ml, open symbols) or application of CTXs alone (6 U/ml, for the three types of IFN, ◇) (see note in Fig. 6 on the way the CTXs were induced). Note the enhancement of cell-killing at low concentrations of IFN as opposed to the protective effect of IFN at higher concentrations (Aderka *et al.*, 1985).

melanoma cells responded to purified LT, and more effectively to LT plus IFN- $\gamma$ , in an arrest at the  $G_0/G_1$  phase (Lee *et al.*, 1984). Indeed, there is preliminary evidence that transcription of the *c-myc* oncogene is in certain tumour cells, effectively turned off by TNF (Yarden, A., Wallach, D. and Kimchi, A. submitted).

## 2 Protective and cell growth promoting effects

Some of the molecular changes observed in cells treated by CTX are inconsistent with the cytotoxic and cytostatic functions of these proteins. Rather than interfering with viable functions of the cells, CTXs have been found to induce an increase in RNA, protein and lipid synthesis (Rosenau, 1980; Ostrove and Gifford, 1979). Furthermore, in cells resistant to the cytotoxic effect of CTXs, these proteins could even be found to stimulate cell multiplication (Sugarman, *et al.*, 1985; Fiers *et al.*, 1986; Vilček *et al.*, 1986). As discussed below, it appears that these "constructive" effects of CTXs in part reflect repair mechanisms that counteract the cytotoxic activity of these proteins, thus preventing non-selective cell destruction. It also seems possible



SV80 cells (A) and in HeLa cells (B), IFN- $\gamma$  was applied at the indicated concentrations prior to infection followed by application of CTX (6 U/ml, 10 U/ml, for the three types of IFN,  $\diamond$ ) (see note on Fig. 5A). Note the enhancement of cell-killing at low concentrations of IFN and the protective effect of IFN at higher concentrations.

and more effectively to LT plus IFN- $\gamma$  (Lee *et al.*, 1984). Indeed, there is evidence that the *c-myc* oncogene is in certain NF- $\kappa$ B (Yarden, A., Wallach, D. and

### effects

in cells treated by CTX are inconclusive. Functions of these proteins. Rather than killing the cells, CTXs have been found to inhibit lipid synthesis (Rosenau, 1980; 1981). In cells resistant to the cytotoxic effect of CTX, it has been found to stimulate cell multiplication (Vilček *et al.*, 1986). As for the "constructive" effects of CTXs in part due to the cytotoxic activity of these proteins. It also seems possible

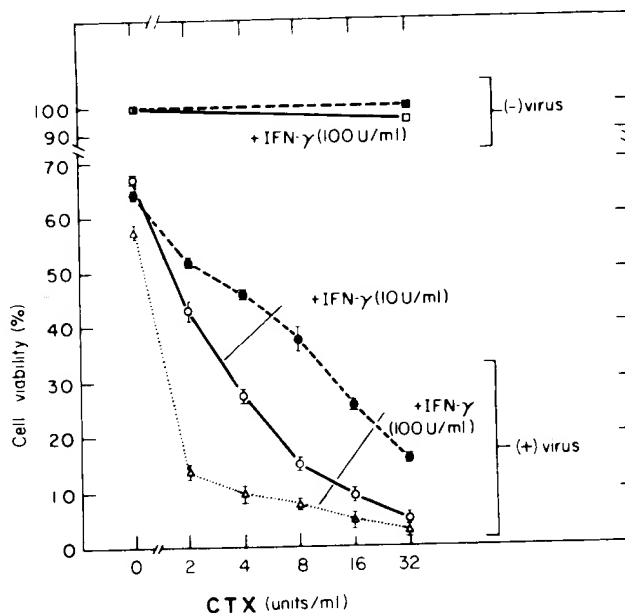


Fig. 6 Enhancement by IFN- $\gamma$  of CTX cytotoxicity in VSV-infected SV80 cells. The cytotoxic effect of CTXs at various concentrations in VSV-infected cells ( $\bullet$ ) and its further enhancement by treating these cells with IFN- $\gamma$  (10 U/ml ( $\circ$ ) or 100 U/ml ( $\Delta$ )) 16 hours prior to infection is shown in comparison to the resistance to the CTXs observed in uninfected cells ( $\blacksquare$ ) even when treated with IFN- $\gamma$  ( $\square$ ). The CTXs used in the experiments described in Figs 5 and 6 were crude preparations of CTXs induced by PHA in PBMC, partially purified by adsorption to controlled pore glass and fully depleted of IFN- $\gamma$  by the use of a monoclonal antibody (Aderka *et al.*, 1985). Selective cytotoxicity against VSV infected cells and its augmentation by IFN could similarly be observed with use of pure TNF.

that these CTX effects contribute to repair on the multicellular level, namely to the increased cell growth necessary for healing the damage in necrotic areas, where at an earlier stage CTX effects have led to the death of cells.

### 3 CTXs as pleiotropic mediators

Apart from effects on cell growth and viability, TNF and LT have recently been found to exert a variety of other effects on cell function. Some of these are immunoregulatory effects; primarily effects on functions related to immune cytotoxicity. LT was reported to induce in granulocytes enhanced ADCC and phagocytic activities (Shalaby, *et al.*, 1985a) and TNF to increase ADCC activity in the U937 histiocytic lymphoma cells (Shalaby, *et al.*, 1985b); it also enhances the production of CTXs by those cells (Table III). There is some evidence that TNF can promote the differentiation of cytotoxic T lymphocytes (cf. Fiers, *et al.*, 1986). Furthermore, it may enhance

cell-killing by cytotoxic T lymphocytes through effects on the target cells: TNF increases the synthesis and cell surface expression of Class I MHC antigens (Collins, *et al.*, 1986) and, according to a preliminary report, also of Class II antigens (Chang and Lee, 1985). Cells that have been exposed to TNF may, therefore, be more effectively recognized by the MHC-restricted cytotoxic T lymphocytes.

Quite a different role for TNF was revealed in the recent identification of TNF with the "cachectin"—a cytokine which suppresses in adipocytes the activity of lipoprotein lipase and also induces in these cells a decrease of mRNAs for some other proteins involved in the differentiated function of the adipocyte (Beutler, *et al.*, 1985a; Torti, *et al.*, 1985). Apparently that effect of TNF contributes to the defence against disease by assuring the availability of lipids for rapid use. However, when induced for prolonged duration the "cachectin" effect of TNF may result in an excessive and dangerous weight loss (cachexia).

That TNF can mediate some deleterious effects in diseases is also indicated by a recent study showing that injected antibodies to TNF reduce in mice the lethal effect of bacterial endotoxins. This indicates that TNF has a causative role in bacterially-induced septic shock (Beutler *et al.*, 1985b).

Since this review was submitted for publication (15 November) until to date (June 1986) many additional effects of TNF on cell functions have been revealed. Quite an unexpected finding, derived both from the studies on TNF and from studies on interleukin-1 (IL-1), is that these two cytokines, which bear no similarity of structure and apparently function through binding to distinct receptors, affect cell function in quite a similar manner. IL-1 can actually be regarded as a cytotoxin—certain tumour cells are killed by IL-1 as effectively as by LT (Onozaki, *et al.*, 1985). IL-1 also shares the ability of TNF to stimulate growth of fibroblasts (Schmidt, *et al.*, 1982). Some other examples of the similarity in function of TNF and IL-1 are: (1) Induction, by both, of the synthesis of collagenase and of prostaglandin  $E_2$ , in fibroblasts (Dayer, *et al.*, 1984, 1985); (2) The "cachectin"-like activity (suppression of lipoprotein lipase in adipocytes) found to be mediated, not only by TNF but also by IL-1 (Beutler and Cerami, 1985); (3) Activation of osteoclasts by the two (Dewhirst, *et al.*, 1985; Bertolini, *et al.*, 1986), and (4) Induction in endothelial cells of increased adhesivity to granulocytes and of synthesis of a cell surface protein which probably is involved in that adherence (Gamble, *et al.*, 1985; Schleimer and Rutledge, 1986; Pober, *et al.*, 1986). Particularly interesting from the point of view of IFN research is the finding that both IL-1 and TNF induce in fibroblasts the synthesis of a specific IFN (IFN- $\beta_2$ ) (Content, *et al.*, 1985; Kohase, *et al.*, 1986). According to preliminary reports, TNF induces synthesis of an IFN also in PBMC (Wong and Goeddel, 1985).

through effects on the target cells: surface expression of Class I MHC (Wright *et al.*, 1983; Williams and Bellanti, 1983; Williamson *et al.*, 1983; Wallach *et al.*, 1983; Stone-Wolff *et al.*, 1984; Lee *et al.*, 1984) as well as with preparations of NKCF (Wright and Bonavida, 1983b; Steinhauer *et al.*, 1985). Furthermore, immunoregulatory effects of TNF and LT were also reported to be potentiated by IFN (Shalaby *et al.*, 1985a, b).

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## B IFN-induced Enhancement of CTX Function

The cytotoxic and cytostatic effects of CTXs and those of IFNs are exerted synergistically. An IFN-induced increase in anticellular activities of CTXs could be observed with crude CTX preparations and with purified TNF and LT (Williams and Bellanti, 1983; Williamson *et al.*, 1983; Wallach *et al.*, 1983; Stone-Wolff *et al.*, 1984; Lee *et al.*, 1984) as well as with preparations of NKCF (Wright and Bonavida, 1983b; Steinhauer *et al.*, 1985). Furthermore, immunoregulatory effects of TNF and LT were also reported to be potentiated by IFN (Shalaby *et al.*, 1985a, b).

Yet, even though IFN appears generally to potentiate the function of CTXs, it has so far not been found to interfere with selectivity in this function by inducing responsiveness to CTXs in cells that in the absence of IFN are completely unresponsive to these proteins. An example of a situation in which IFN potentiates CTX activity without decreasing its selectivity is presented in Figs 5 and 6 in which the cytotoxic effect of IFN-free CTX preparations in SV-80 and HeLa cells is quantitatively analysed, comparing cells that were infected by VSV to uninfected cells. Under normal growth conditions the cells are quite resistant to killing by CTXs but they do respond to the cytotoxic effect following infection by the virus (cf. Fig. 6, curves ■ and ●). IFN is found to potentiate killing of the infected cells but not to impose vulnerability to this cytotoxic effect on the uninfected cells (cf. Fig. 6, curves □ and △). Since, at antiviral concentrations, IFN suppressed replication of the virus, an IFN-induced increase in killing of the infected cells could only be observed at sub-antiviral concentrations. At higher IFN concentrations, suppression of virus replication by IFN resulted in resurgence of the resistance to the effect of the CTXs (compare, in Fig. 5B, the enhancement of killing of HeLa cells by IFN- $\gamma$  at 0.01 U/ml to the protection from killing when the IFN concentration was 10 U/ml).

## C Mechanisms Involved

Radioactively tagged TNF and LT bind to high-affinity sites on the surface of cells; the number of binding sites range from undetectable levels to as much as 18,000 per cell, depending on the cell line (Hass *et al.*, 1985; Kull *et al.*, 1985; Tsujimoto *et al.*, 1985, 1986; Israel *et al.*, 1986). Using bifunctional cross-linking agents, specific cell surface proteins could be observed to which TNF bind, forming conjugates with  $M_r$  of about 75 and 92 Kd (Fig. 7 and Kull *et al.*, 1985).

These findings provide direct evidence for the long-suspected existence of cell surface receptors for CTXs. A clear distinction can now be made between those CTXs that function through binding to specific receptors and other



Fig. 7 Characterization of the receptors to human TNF on human U937 (a, b) and KG-1 (c-e) cells by their cross-linking to  $^{125}\text{I}$ -TNF. a, b, e: cross-linking with disuccinimidyl suberate (in b-excess nonlabelled TNF was included during incubation with the labelled protein). c, d: cross-linking with the bifunctional agent dithiobis succinimidyl propionate which is cleavable with  $\beta$ -mercaptoethanol; analysis in the absence (c) and presence (d) of  $\beta$ -mercaptoethanol. Cells were incubated for 2 hours with  $^{125}\text{I}$ -TNF (100 U/ml), which was radio-iodinated by the chloramine-T method. They were then washed, treated with the cross-linking agents and extracted with a buffer containing 2% Triton X-100. The extracts were analysed by SDS/7.5%-PAGE followed by autoradiography. The heavy radioactive band at the bottom of lane a is free TNF (Israel *et al.*, 1986).

cytotoxic proteins, also present in crude CTX preparations, that kill cells via enzymic activity (e.g. arginase or protease (Currie, 1978; Adams, 1980)). A third mode of cell-killing by leukocyte-produced proteins is exemplified in the function of the 'perforins' (see Section II (B)) which, like the  $\text{C}_9$  component of the complement, are believed to kill cells by forming channel-like structures in cell membranes, apparently with no prior interaction with any receptor protein.

The nature of the mechanisms activated by occupation of the TNF receptor, and which eventually lead to the functional changes induced by the CTXs, remains obscure. Similarly to cell surface receptors for many other agonists, the receptors for TNF are taken up into the cell after becoming occupied and the CTXs bound to these receptors are then degraded, apparently in lysosomes (Tsujimoto *et al.*, 1985). Clearly this uptake process has no role in the cytotoxic effect, as cytotoxicity can be effectively induced in the presence of sodium azide, which fully blocks the uptake (unpublished results). Neither does it appear that the cytotoxic effect is dependent on





TNF on human U937 (a, b) and KG-1 (c, e) cross-linking with disuccinimidyl suberate (in incubation with the labelled protein). c, d: succinimidyl propionate which is cleavable and presence (d) of  $\beta$ -mercaptoethanol. Cells (2 ml), which was radio-iodinated by the treated with the cross-linking agents and The extracts were analysed by SDS 7.5% radioactive band at the bottom of lane a is free

TX preparations, that kill cells via (Currie, 1978; Adams, 1980)). A reduced proteins is exemplified in section II (B)) which, like the C<sub>0</sub>, led to kill cells by forming channel-ly with no prior interaction with

l by occupation of the TNF recep- functional changes induced by the surface receptors for many other on up into the cell after becoming eceptors are then degraded, appar- 5). Clearly this uptake process has ity can be effectively induced in the blocks the uptake (unpublished : cytotoxic effect is dependent on

synthesis of any protein or RNA in the target cell, since it occurs in the presence of azide, CHI or actinomycin D; these agents actually potentiate the cytotoxic effect of CTXs. The fact that the cytotoxic effect of CTXs is exerted in the presence of inhibitors of RNA, protein and ATP synthesis, also implies that the CTXs themselves do not possess such a general inhibitory capacity and that their cytotoxic function reflects another mode of action. Specific RNA and protein species do decrease in response to TNF, apparently owing to suppressed synthesis of these molecules (Torti *et al.*, 1985), but TNF and LT have also been found to induce an increase in the synthesis of cellular RNA, proteins and lipids (Rosenau 1980; Ostrove and Gifford 1979).

Thus, as in the function of various other regulatory cytokines, we are faced in the function of CTXs with transduction of information from the receptors for these proteins to the interior of the cell, in a so far unknown fashion, resulting in altered synthesis of specific species of RNA and protein molecules and yet those effects are apparently distinct from those participating in the cytotoxic effect of the CTXs. Actually, as mentioned above, it has been suggested that the enhancement of synthesis of RNA, protein and lipid by CTXs has a role in counteracting the cytotoxic effects which the CTXs themselves induce (Rosenau, 1980). The fact that metabolic inhibitors such as CHI and actinomycin D, which are likely to interfere with CTX-induced increases in anabolic activities, sensitize cells to the cytotoxic effect, is consistent with a protective role for these CTX-induced changes. In further support for the idea that CTXs activate mechanisms in cells that counteract their cytotoxicity, we have found that, within a short time after application of CTXs, cells become less responsive to sensitization by CHI (Fig. 8).

Such induction of resistance to CTX by the CTX itself could be observed with purified TNF (Hahn *et al.*, 1985) as well as with partially purified preparations of LT, free of TNF, the two kinds of preparations inducing mutual resistance as well as resistance to their own effect (unpublished observations). Auto-induction of resistance to TNF could not be related to depletion of receptors to the protein. As shown in Fig. 9, within a few hours of removal of TNF, which had been applied to the cell at saturating concentrations, free cell-surface receptors for the protein were replenished, yet the extent of resistance to TNF cytotoxicity induced by such pretreatment remained unchanged. It therefore seems likely that this resistance indeed reflects the activation by TNF of mechanisms that counteract its cytotoxic effects.

Recently we have noticed that, besides TNF and LT, several other cytokines, including insulin and interleukin-1 (IL-1) can induce in cells resistance to the cytotoxicity of the CTXs. Further examination of the protective effect of IL-1 revealed induction, by this cytokine, of a rapid and effective decrease in cell surface receptors to TNF. That decrease could not fully account for

the protective effect of IL-1 since following the removal of IL-1 TNF receptors were rapidly replenished yet the cells remained resistant to the cytotoxicity of TNF (Holtmann, Hahn and Wallach, submitted).

The potentiation of CTX effects by IFN can be related in part to an IFN-induced increase in the number of CTX receptors. As shown in Table IV, cells of several lines show an increase in binding of  $^{125}\text{I}$ -TNF following treatment with IFN- $\gamma$ . Some of these cells also display such an increase when treated by IFN- $\alpha$ . An IFN-induced increase in binding of TNF has also been recently observed by others (Tsujimoto *et al.*, 1986; Aggarwal *et al.*, 1985b and Ruggiero *et al.*, 1986). These studies indicate that this IFN effect reflects an increase in amounts of the receptor protein, apparently as a consequence of its increased synthesis. It is unlikely, though, that this moderate IFN-induced increase in receptor density fully accounts for the pronounced potentiation of CTX function by IFN (compare the increase in receptor level to cytotoxic effect in Fig. 10). Other IFN-induced changes are probably also involved. Perhaps the increased sensitivity of IFN-treated cells to the cytotoxic effect of CTXs reflects not only potentiation of the mechanisms mediating this effect but also suppression of cellular mechanisms that counteract it. Consistent with this idea, the induction of auto-resistance to

TABLE IV IFN-induced increase in receptors for TNF.

Cells	IFN (1000 U/ml)	CTX Binding	
		(cpm/ $10^6$ cells)	(relative increase)
HeLa	—	1080	1
	IFN- $\alpha$	1800	1.67
	IFN- $\gamma$	2200	2.04
WISH	—	1050	1
	IFN- $\alpha$	1000	0.95
	IFN- $\gamma$	1440	1.37
L-132	—	800	1
	IFN- $\alpha$	1000	1.25
	IFN- $\gamma$	1040	1.3
FS11	—	440	1
	IFN- $\alpha$	400	0.91
	IFN- $\gamma$	650	1.48
U937	—	920	1
	IFN- $\alpha$	1300	1.41

Specific binding of radiolabelled TNF at a concentration of 2 U/ml (U937 cells) and 10 U/ml (other cells) was determined following 12 hours treatment by rIFN- $\alpha$  or rIFN- $\gamma$  at 1000 U/ml. (Israel *et al.*, 1985).

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 lls remained resistant to the cyto-  
 Wallach, submitted).  
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1040	1.3
440	1
400	0.91
650	1.48
920	1
1300	1.41

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 atment by rIFN- $\alpha$  or rIFN- $\gamma$  at 1000 U ml.

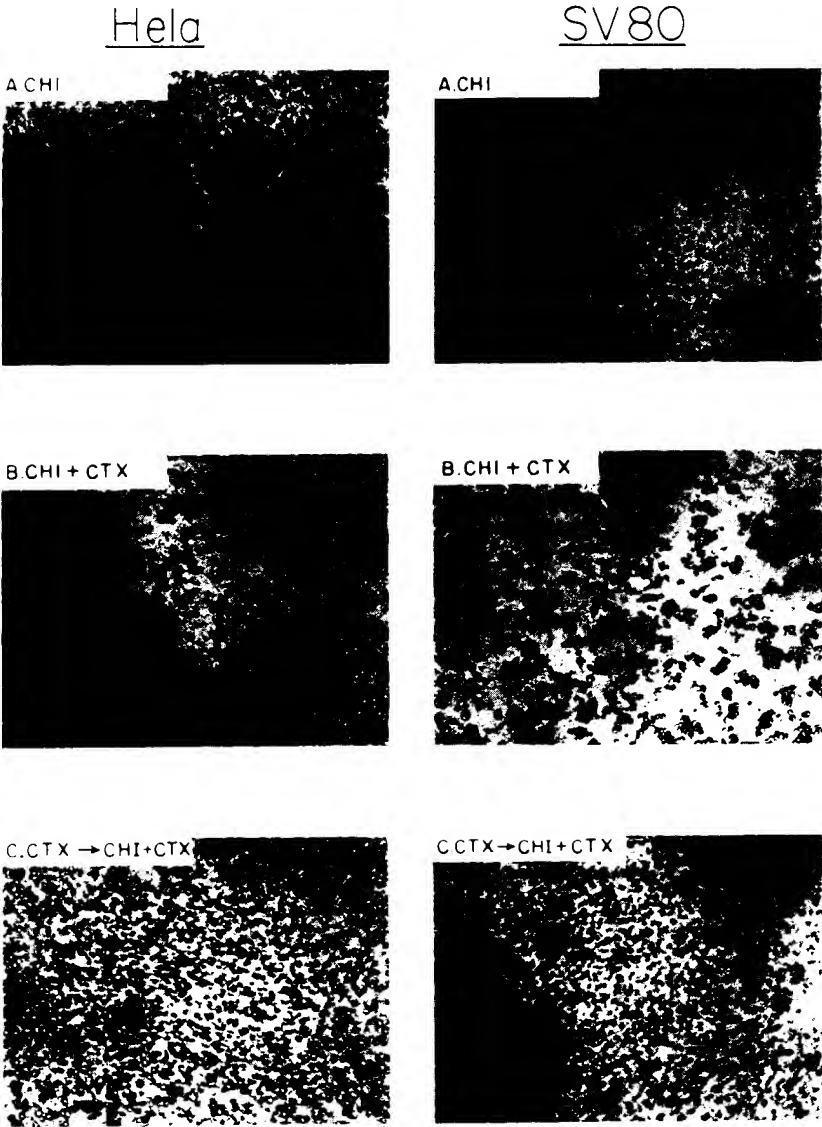


Fig. 8 Auto-induction of resistance to the cytotoxic effect of CTXs. The killing of HeLa and SV80 cells by a crude preparation of PHA-induced human CTXs (8 U/ml), incubated for 12 hours together with CHI (50  $\mu$ g/ml) (B), compared with the normal morphology of the cells observed on incubation with CHI alone (A). The extent of killing is markedly reduced in cells which were pretreated for 3 hours with CTXs (80 U/ml) in the absence of CHI, rinsed and treated again with CTX (8 U/ml) this time in the presence of CHI (C) (Wallach, 1984).

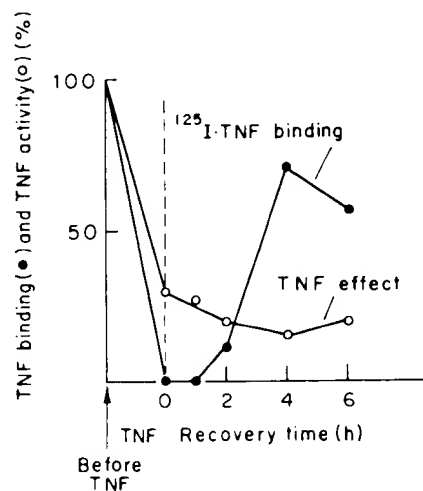


Fig. 9 Lack of correlation between TNF receptor concentration and decrease in vulnerability to its cytotoxic effect following pretreatment of L132 human cells with TNF. L132 human cells were incubated for 2 hours with TNF (260 U/ml), rinsed and further incubated in TNF-free medium. At various times the ability of the cells to bind TNF and to respond to its cytotoxic effect was measured; the former by binding of  $^{125}\text{I}$ -labelled TNF (60 U/ml) and the latter by measuring the cytotoxicity of TNF (7 U/ml) in the presence of CHI. Results are presented as per cent of TNF bound specifically (960 c.p.m.  $10^5$  cells) and of the cytotoxic titre of the test sample of TNF, observed before pretreatment by TNF (Israel *et al.*, 1986).

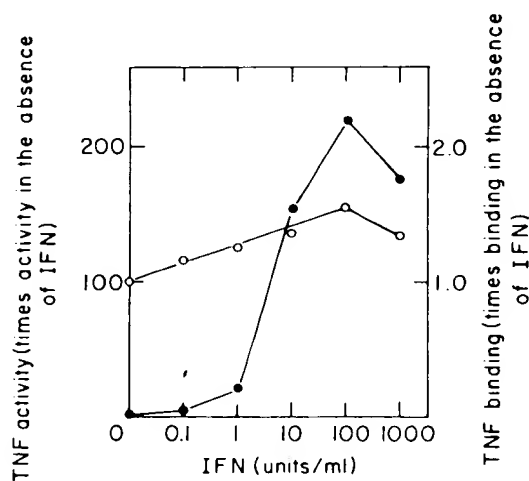


Fig. 10 Titration of the effect of IFN- $\gamma$  on the cytotoxic activity of human TNF (solid symbols) and on the binding of the protein to its receptors (open symbols) in human HeLa cells. Cells were incubated for 12 hours with the indicated concentrations of IFN- $\gamma$ . Their vulnerability to the cytotoxic effect of TNF was then determined by titrating; in the presence of CHI, the cytotoxic activity of a given preparation of TNF (titre of 530 U/ml in cells not treated by IFN). Binding of TNF was determined by incubating the cells with saturating levels of  $^{125}\text{I}$ -TNF (7500 U/ml). In cells not treated by IFN, specific binding of 1000 c.p.m.  $10^5$  cells was observed (Israel *et al.*, 1986).

TNF, described above, is found to be greatly reduced in cells treated by IFN (unpublished results).

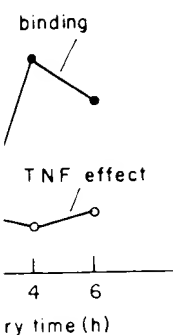
## V ON THE PHYSIOLOGICAL ROLE OF THE CTXs

There is little information available on the formation and function *in vivo* of CTXs. The physiological role of these proteins can, therefore, only be a matter of speculation, on the basis of our present fragmentary knowledge of the way these proteins are formed and function *in vitro*. The following implications of that knowledge seem to me particularly worthy of emphasis.

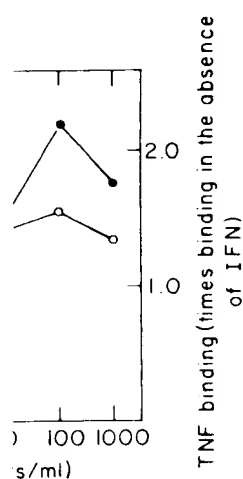
(1) It seems reasonable to assume that the CTXs function against their inducing agents; these are found to be quite heterogeneous. Thus, a bacterial product (LPS), a virus (Sendai virus), a T cell mitogenic agent (PHA), and tumour cells have all been found to induce TNF. Like the IFNs, CTXs may, therefore, play a rather non-specific role in the immune response, contributing to the defence against a variety of pathogenic agents. Indeed, one may infer a heterogeneity of functions for CTXs from the way they affect cells: a role for TNF in the immune response to viral infection may be indicated by the increased vulnerability to its cytotoxic effect in cells infected by certain viruses. An antibacterial role is perhaps indicated by the augmentation of phagocytic activity of granulocytes by TNF and an anti-tumour role by its cytotoxic effect against certain tumour cell lines.

(2) Some of the leukocyte-mediated cytotoxic activities (such as that of the cytotoxic T lymphocytes) are found to be exerted solely against those target cells to which these leukocytes bind. Involvement of CTXs in such "contact-dependent" cytotoxic activities has often been suggested, but remains to be confirmed. Even if it turns out that TNF or LT do take part, it is unlikely to be their sole function. The fact that those proteins can be induced by both cellular and non-cellular inducers (such as LPS) and the way the CTXs function, by binding avidly to cell surface receptors, which should allow them to affect remote target cells, strongly suggest that TNF and LT have a role as mediators which function distantly from their producing cells.

(3) We might understand the function of CTXs better if we bear in mind that these proteins are likely to be produced in areas of inflammation; specifically in inflammation reflected in delayed-type hypersensitivity (DTH). Indeed, among the T lymphocytes, the ones believed to be most involved in DTH (the  $\text{Lyt } 1^+$  cells) are those found to produce the CTXs (Eardley *et al.*, 1980; Tite *et al.*, 1985). In the micro-environment formed by "walling off" the inflammatory area as a result of lymphatic blockage, CTXs may function more effectively. They may reach a high concentration



concentration and decrease in vulnerability of L132 human cells with TNF. L132 human cells were rinsed and further incubated in TNF-free medium to bind TNF and to respond to its cytotoxic effect. The results are presented at per cent of the cytotoxic titre of the test sample (Israel *et al.*, 1986).



cytotoxic activity of human TNF (solid symbols) and of Sendai virus (open symbols) in human HeLa cells. Cells were incubated in the presence of IFN- $\gamma$ . Their vulnerability to the cytotoxic effect of TNF, in the presence of CHI, the cytotoxic effect of TNF (7500 U/ml) in cells not treated by IFN. Binding of saturating levels of  $^{125}\text{I}$ -TNF (7500 U/ml). 10<sup>5</sup> c.p.m. 10<sup>5</sup> cells was observed (Israel *et al.*, 1986).

along with IFNs produced *in situ* which can potentiate the actions of the CTXs. The cytotoxic functions of the CTXs, found to be greatly increased at elevated temperatures *in vitro* (Peter *et al.*, 1973; Ruff and Gifford, 1981b) can be potentiated by the fever in the area of inflammation. Limited supply of nutrients and of oxygen to this region may sensitize cells to the cytotoxic effect of CTXs, similarly to the sensitization observed *in vitro* when applying metabolic blockers to cells. Accumulation of MP and of granulocytes at the site of delayed type hypersensitivity, where CTXs are likely to be formed, should allow these cells to respond to the immunoregulatory effects of the CTXs. In addition, response of remote tissues to the CTXs leaking into the circulation from the site of inflammation, such as the inhibition of lipoprotein-lipase by TNF, may contribute to the defence at the level of the whole organism. Unfortunately, most information on the role of DTH reactions relates to the pathological implications of this inflammatory response (tissue damage caused in viral infection, graft rejection, etc.). This information may provide clues to the nature of situations in which CTX formation is deleterious, but gives little idea of what positive functions the CTXs can fulfil.

(4) Although the function of CTXs is not antigen-specific, there does appear to be some selectivity in their effects. That selectivity is dictated firstly by dependence of the function of CTXs (at least the function of TNF and LT) on cell surface receptors to those proteins. While the amounts of these receptors are known to vary, there is no information yet on the physiological parameters that control the variation, other than the fact that some increase in the receptor level can be induced by IFN. (As mentioned above (see page 113) we have recently noted that TNF receptors are subjected also to effective modulation by interleukin-1. Unlike the IFN-induced increase in TNF receptors which appears to reflect increased synthesis of the receptor protein, the IL-1 induced decrease in receptors for TNF is independent of protein synthesis, and probably reflects uptake or inactivation of the receptors (Holtmann and Wallach, submitted).) Further selectivity may be imposed on the function of CTXs by the dependence of their cytotoxic effect on a prior sensitization of the affected cell. Although the biochemical nature of the sensitization mechanism is not known, it appears, by the nature of those agents known to sensitize cells (metabolic blockers, viruses and x-radiation), that it is due to interference with some vital functions of the cell. CTXs can thus be viewed as agents with the capacity for distinguishing between "healthy" cells, which can resist their cytotoxicity and thus be spared from killing, and "sick" cells, which fail to resist CTX cytotoxicity and are therefore specifically eliminated.

(5) At present, there is no solid evidence for a specific anti-tumour role of CTXs, linking vulnerability of the tumour cell to CTXs with a specific

ich can potentiate the actions of CTXs, found to be greatly increased *al.*, 1973; Ruff and Gifford, 1981b) ie area of inflammation. Limited is region may sensitize cells to the the sensitization observed *in vitro* cells. Accumulation of MP and of pe hypersensitivity, where CTXs se cells to respond to the immunon, response of remote tissues to rom the site of inflammation, such TNF, may contribute to the defence rtunately, most information on the pathological implications of this aused in viral infection, graft rejec- e clues to the nature of situations in ut gives little idea of what positive

characteristic of malignancy—for example, the growth of the cells independently of supply of growth-factors or the activation of specific oncogenes. Yet the mere fact that some kinds of tumour cells respond to the cytotoxic or cytostatic effects of CTXs, even though they have failed to respond to mechanisms that normally restrain cell growth, is potentially applicable in tumour therapy (see Rundell and Evans, 1981; Ruff and Gifford, 1981a; Lee *et al.*, 1984). Perhaps it will be possible to direct CTX activity selectively against tumour cells, taking advantage of the fact that DNA-intercalating drugs and ionizing radiation, which preferentially affect rapidly-growing tumour cells, sensitize cells to the cytotoxic effect of CTXs. From the point of view of IFN research, the most intriguing question is whether effects of CTXs on tumour cells and their synergism with IFN can contribute to increasing the effectiveness of IFN in tumour therapy: do CTXs produced *in situ*, formed in increased amounts owing to the enhancement of CTX formation by IFN, contribute to some of the anti-tumour effects that IFNs have already been found to exert, and can CTXs, injected into patients together with IFN, further potentiate these anti-tumour effects?

## VI CONCLUDING REMARKS

While there is some recent information on the structure of CTXs, our knowledge of their function, the mechanisms involved, and their physiological role is still quite limited. Basically what has been learned in the short time that purified CTXs have been available is that the way in which CTXs function is more complex than was believed hitherto. The cytotoxic activity of the CTXs had been thought to be non-specific, leading to killing of "innocent bystander cells". A more thorough examination now shows that this activity can be subjected to effective modulation, depending on the nature of the target cell and its metabolic state. Furthermore, when they do not exert cytotoxic effects, CTXs mediate other regulatory functions.

Which of the various effects of CTX function observed *in vitro* leads to physiologically meaningful consequences is not known. Nor can we tell yet whether the practical benefits of these proteins, such as the killing of tumour cells synergistically with IFN, will be found sufficiently effective to be applicable in therapy. Perhaps it will be the adverse effects, such as mediation of cachexia or of shock symptoms in disease, that will turn out to be the more prominent, necessitating a search for means of suppressing CTX activity. We know that TNF and LT do not act simply by exerting an enzymatic activity but rather by binding to specific receptors, thus activating cellular mechanisms that affect gene regulation and can also mediate cell death. The nature of those mechanisms is yet to be elucidated. Knowledge of the mechanisms

is not antigen-specific, there does ets. That selectivity is dictated firstly at least the function of TNF and LT) teins. While the amounts of these information yet on the physiological her than the fact that some increase FN. (As mentioned above (see page eptors are subjected also to effective IFN-induced increase in TNF recep- ynthesis of the receptor protein, the TNF is independent of protein syn- inactivation of the receptors (Holt- r selectivity may be imposed on the of their cytotoxic effect on a prior ough the biochemical nature of the it appears, by the nature of those ic blockers, viruses and x-radiation), vital functions of the cell. CTXs can for distinguishing between "healthy" and thus be spared from killing, and ototoxicity and are therefore specifi-

nce for a specific anti-tumour role of our cell to CTXs with a specific

initiating synthesis of CTXs within the CTX-producing cells is also quite limited.

Our present incomplete state of knowledge of the CTXs provides intriguing hints toward a deeper understanding in the future. The lack of resemblance between the TNFs (TNF and LT) and other known proteins; the way these proteins kill cells; the presence of receptors to TNF and LT on many differing cells; the ability of TNF and LT to initiate cellular responses other than cell death; and the multiplicity of agents that induce production of these proteins, all indicate that further studies on these proteins, as well as of the way their formation and function are regulated by IFN, may disclose novel information on mechanisms controlling the life and death of cells and on ways whereby the immune response can regulate these mechanisms.

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## Processing of Newly Synthesized Cachectin/Tumor Necrosis Factor in Endotoxin-Stimulated Macrophages<sup>†</sup>

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**ABSTRACT:** The biosynthesis and processing of cachectin/tumor necrosis factor (TNF) were examined in the murine macrophage-like cell line RAW 264.7. Lipopolysaccharide-stimulated cells secreted both glycosylated and nonglycosylated 17-kilodalton (kDa) mature cachectin/TNF into the culture medium. Secreted cachectin/TNF was derived from membrane-associated precursors that were precipitated by polyclonal antisera raised against either the mature protein or synthetic peptide fragments of the 79 amino acid cachectin/TNF prohormone sequence. About half of the precursors were N-glycosylated, apparently cotranslationally. The cachectin/TNF precursors were then proteolytically cleaved to release soluble mature cytokine into the medium, while the membrane-bound 14-kDa prosequence remained cell associated. During the period of LPS stimulation, the amount of macrophage cell surface cachectin/TNF remained at a low level, suggesting that both nonglycosylated and glycosylated precursors of cachectin/TNF are efficiently cleaved by these cells. These findings suggest the presence of a unique mechanism for the secretion of cachectin/TNF.

Microbial and parasitic infections and neoplastic diseases in mammals alter the physiological and metabolic state of the host and can advance to cachexia and septic shock (Beisel, 1975). Recently, macrophage-derived polypeptide cytokines (monokines), especially cachectin/tumor necrosis factor (TNF)<sup>1</sup> and interleukin 1 (IL-1), have been implicated as mediators of such metabolic changes in infected hosts [reviewed by Beutler and Cerami (1987) and Dinarello (1984)]. When stimulated by bacterial endotoxin, macrophages secrete large amounts of these cytokines. Cachectin/TNF has been found to mediate catabolic responses in septic animals and to be responsible for endotoxin-induced injury and death (Tracey et al., 1986). Cachectin/TNF has other known *in vivo* and *in vitro* effects on tumor cells (Carswell et al., 1975) and participates in host inflammatory responses to viral, bacterial, and parasitic stimuli (Beutler & Cerami, 1987).

Cloning of cDNA for full-length cachectin/TNF mRNA and comparison of its coding sequence with that of the mature, secreted, 17 kDa hormone had revealed that cachectin/TNF is synthesized as a prohormone, whose prosequence is so long that it has not been regarded as a typical "signal" sequence: 76 and 79 amino acid residues for human (Pennica et al., 1984; Shirai et al., 1985) and murine (Fransen et al., 1985; Pennica et al., 1985; Caput et al., 1985) cachectin/TNFs, respectively. The propeptide sequence is highly conserved (86% homologous) between human and mouse proteins, which raised the

possibility that it serves a distinct biological function (Beutler & Cerami, 1987). The prosequence has a centrally located hydrophobic region and in this respect resembles other secretory signal sequences (Blobel et al., 1979). However, recent reports indicate unusual features in posttranslational processing of cachectin/TNF as a secretory protein (Muller et al., 1986; Decker et al., 1987; Kriegler et al., 1988). In human monocytes and cells transfected with a cachectin/TNF prohormone cDNA construct, the intact cachectin/TNF prohormone remains associated with the membrane fraction. It was postulated that this long form is clipped to release mature 17-kDa cachectin/TNF into the medium (Muller et al., 1986; Kriegler et al., 1988).

In the present study we investigated the processing of cachectin/TNF in an endotoxin-stimulated murine macrophage-like cell line. Cachectin/TNF was produced initially as a membrane-bound, cell-associated, 26-kDa precursor that was then cleaved to yield soluble, mature, 17-kDa protein, while 14-kDa prosequence peptide remained membrane bound.

### EXPERIMENTAL PROCEDURES

**Cell Culture and *In Vitro* Cytotoxicity Assay.** RAW 264.7 murine macrophage and L-929 mouse fibroblast lines were obtained from American Type Culture Collection (Rockville, MD). RAW 264.7 cells were grown in RPMI 1640 medium

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<sup>1</sup> Abbreviations: TNF, tumor necrosis factor; IL-1, interleukin 1; HEPES, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; LPS, lipopolysaccharide; D-PRIS, Dulbecco's phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

(Gibco, Grand Island, NY) supplemented with 20 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES, Research Organics, Cleveland, OH), 50  $\mu$ g/mL gentamycin (Gibco), and 10% heat-inactivated (56 °C, 30 min) fetal bovine serum (FBS, Hyclone, Logan, UT) (RPMI/F10).

For the *in vitro* cytotoxicity assay, L-929 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 50  $\mu$ g/mL gentamycin and 10% calf serum (Hyclone) (DMEM/C10). The assay was performed as described previously (Ostrove & Gifford, 1979) with slight modifications. L-929 cells at a density of 30 000 cells per well in 96-well plates were incubated at 37 °C for 24 h before the addition of cachectin/TNF and 1  $\mu$ g of actinomycin D/mL. After 16 h, viable cells were stained with a chromogen, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma Chemical Co., St. Louis, MO) and optical densities at 570/690 nm were measured by using an automated enzyme-linked immunosorbent assay (ELISA) plate reader (Bio-Tek Instruments, Burlington VT) (Moxmann, 1983; Wolpe et al., 1988).

**Antisera.** Polyclonal antisera to murine cachectin/TNF were prepared by immunizing rabbits with purified 17-kDa cachectin/TNF (B. Sherry, D.-M. Jue, A. Zentella, and A. Cerami, unpublished result). Rabbit antibodies to portions of the prosequence of cachectin/TNF were raised against three synthetic peptides that correspond to different portions of the murine prosequence (residues 1–11, 47–67, and 68–77 in procachectin/TNF). Each peptide was conjugated to bovine thyroglobulin (Sigma) by using glutaraldehyde (Sigma) (molar ratio of peptide to carrier protein, 10:1) and 250  $\mu$ g of peptide-thyroglobulin conjugate was injected into rabbits for immunization (B. Sherry, D.-M. Jue, A. Zentella, and A. Cerami, unpublished result). All antisera against prosequence peptides gave qualitatively similar results in immunoprecipitations and a mixture of equal volumes of these antiprosequence antisera was used for immunoprecipitations.

**Cell Stimulation and Metabolic Labeling.** RAW 264.7 cells grown to confluence in six-well plates (Linbro, Flow Laboratories, McLean, VA) were treated with 1  $\mu$ g of lipopolysaccharide (LPS, LPS W, *E. coli* 0127:B8, Difco Laboratories, Inc., Detroit, MI) per milliliter in RPMI/F10. After incubation for 2 h at 37 °C, the cells were washed twice with warm methionine-free DMEM supplemented with 2 mM L-glutamine (Gibco) (labeling medium) and incubated for 30 min in the same medium containing the stimulants. The medium was then replaced with 0.5 mL of labeling medium containing 50  $\mu$ Ci [<sup>35</sup>S]methionine (New England Nuclear, Boston, MA) and the cells were incubated for another 30 min. In some samples tunicamycin (Sigma) was added to the cells at a concentration of 10  $\mu$ g/mL in both stimulation and labeling media. Tunicamycin was dissolved in RPMI/F10 adjusted to pH 9.0. After the supernatant was harvested, the cells were washed with ice-cold Dulbecco's phosphate-buffered saline (D-PBS) three times and then lysed in 0.5 mL of 50 mM Tris-HCl (pH 7.4), 0.5% Nonidet P-40 (NP40), 0.15 M NaCl, 0.02% sodium azide, 5 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM phenylmethanesulfonyl fluoride (PMSF), soybean trypsin inhibitor (20  $\mu$ g/mL) (Sigma), 5  $\mu$ M leupeptin (Sigma), and 5  $\mu$ M antipain (Sigma) (lysis buffer). After 10 min, cell lysate was removed, vortexed, and clarified by centrifugation. Reserved culture medium was centrifuged and 50  $\mu$ L of 10-fold-concentrated lysis buffer was added. For pulse-chase experiments, cells were labeled for various times and the medium was changed with complete DMEM. At various times after the medium change, cells and culture

medium were harvested and treated as described above. The culture media of the remaining samples were also changed with fresh ones in order to measure the secretion of cachectin/TNF during certain periods of time.

**Immunoprecipitation and Gel Electrophoresis.** Cell lysates and culture supernatants were incubated with 10  $\mu$ L of 50% protein A Sepharose (Pharmacia, Piscataway, NJ) suspension at 22 °C for 1 h. The Sepharose beads were removed by centrifugation and 40  $\mu$ L of rabbit antisera against mature cachectin/TNF or prosequence fragment conjugates was added. After incubation for 16 h at 4 °C, 80  $\mu$ L of 50% protein A Sepharose bead suspension was added and the mixture was incubated for another 2 h at 4 °C. All incubations were performed with gentle agitation. The Sepharose beads were washed twice each with lysis buffer, D-PBS containing 0.05% sodium azide, and then with 0.1% sodium dodecyl sulfate (SDS). The washed beads were combined with 50  $\mu$ L of 2X sample buffer (final concentration, 62.5 mM Tris-HCl, pH 6.9, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol) and heated at 90 °C for 5 min. The samples were analyzed in denaturing 12.5–18% gradient polyacrylamide gels as described previously (Laemmli, 1970). Gels were fixed with 35% methanol/10% acetic acid, treated with Autofluor (National Diagnostics, Manville, NJ), dried, and used to expose Kodak XAR-5 film at -70 °C.

**Digestion with Glycosidic Enzymes.** [<sup>35</sup>S]Methionine-labeled cells were immunoprecipitated and the beads were washed as described above. Fifty microliters of 0.2 M sodium phosphate buffer (pH 6.0), 0.2% SDS, and 2% 2-mercaptoethanol were added and the samples were heated for 5 min at 95 °C. After the addition of 10  $\mu$ L of 10% NP40 and 10 mM PMSF, the samples were incubated at 37 °C for 12 h with 250 milliunits of endoglycosidase F (endo- $\beta$ -*N*-acetylglucosaminidase F, Boehringer Mannheim Biochemicals, Indianapolis, IN), 100 milliunits of neuraminidase (Sigma, type II), and/or 3 milliunits of  $\alpha$ -glycanase (endo- $\alpha$ -*N*-acetylglucosaminidase, Boehringer Mannheim Biochemicals). Digestions were stopped by the addition of 100  $\mu$ L of 2X electrophoresis sample buffer before analysis by gel electrophoresis.

**Fractionation of RAW 264.7 Cells Into Cytosol and Membrane/Particulate Fractions.** LPS-stimulated RAW 264.7 cells were labeled with [<sup>35</sup>S]methionine as described above. The cells were washed three times with ice-cold D-PBS, harvested with a cell scraper (Gibco), and collected by centrifugation. Hypotonic swelling and homogenization of the cells were performed as described by Matsushima et al. (1986) except that soybean trypsin inhibitor (20  $\mu$ g/mL), 5  $\mu$ M leupeptin, and 5  $\mu$ M antipain were added to the hypotonic buffer. The nuclei and undisturbed cells were removed by centrifugation at 200g for 10 min and the supernatant was centrifuged again at 100 000g for 60 min at 4 °C. After centrifugation the supernatant was used as the cytosol fraction and the pellet as the membrane/particulate fraction. Each was combined with lysis buffer and analyzed by immunoprecipitation and gel electrophoresis.

**Determination of Cell Surface Cachectin/TNF.** RAW 264.7 cells were grown to confluence in six-well plates and stimulated with LPS. At 1, 2, 4, 8, and 16 h after LPS addition, culture media were harvested and the cells were washed with warm D-PBS three times. The cells were fixed by addition of 1 mL of 1% paraformaldehyde (Sigma) in D-PBS. The plate was incubated for 30 min at 4 °C with gentle shaking. Fixed cells were washed three times with D-PBS and 2 mL of fresh RPMI/F10 was added. After



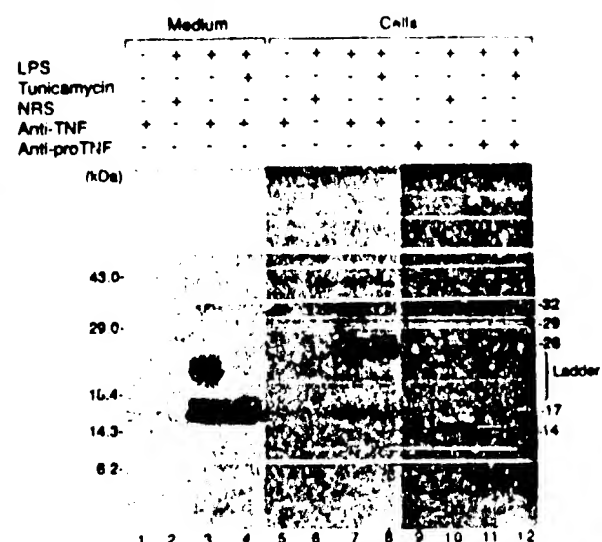


FIGURE 1: Immunoprecipitation analysis of cachectin/TNF produced by RAW 264.7 cells. Cells were incubated in the presence (lanes 2-4, 6-8, and 10-12) or absence (lanes 1, 5, and 9) of LPS for 2.5 h and then labeled with [ $^{35}$ S]methionine for 30 min. Culture media (lanes 1-4) and cell lysates (lanes 5-12) were immunoprecipitated with anti-mature cachectin/TNF antisera (lanes 1, 3-5, 7, and 8), anti-propeptide fragment antisera (lanes 9, 11, and 12), or normal rabbit sera (NRS) (lanes 2, 6, and 10) and analyzed by electrophoresis. Lanes 4, 8, and 12 are from cells treated with tunicamycin during incubation with LPS and labeling. The positions of molecular weight markers are shown on the left, and cachectin/TNF immunoreactive bands are identified by their apparent molecular weights at right.

incubation at 37 °C for 24 h, the cells were scraped off the plate, washed again with fresh media, and resuspended in 0.5 mL of RPMI/F10. Serial dilutions of these cells were added to monolayers of L-929 cells to measure cytotoxic activity.

## RESULTS

**Glycosylation of Cachectin/TNF.** RAW 264.7 cells were stimulated for 2.5 h in LPS-containing medium and metabolically labeled with [ $^{35}$ S]methionine for another 30 min. When the medium was analyzed by immunoprecipitation with anticachectin/TNF antiserum and electrophoresis, mature 17-kDa cachectin/TNF and related cachectin/TNF "ladder" proteins were observed (Figure 1, lane 3). As previously observed in this laboratory (B. Sherry, D.-M. Jue, A. Zentella, and A. Cerami, unpublished results), the synthesis of "ladder" proteins was blocked when the cells were cultured in the presence of tunicamycin, an inhibitor of asparagine (N)-glycosylation (Takatsuki & Tamura, 1971). Tunicamycin treatment did not significantly affect the total amount of secreted cachectin/TNF. When the mixture of secreted cachectin/TNF was treated with *N*-glycanase to remove N-linked carbohydrate groups (Elder & Alexander, 1982), most of the cachectin/TNF "ladder" proteins disappeared and the intensity of the 17-kDa band increased (Figure 2, lane 6). However, one of the "ladder" proteins with apparent molecular weight of 18.5 kDa was found to be resistant to both treatments (Figures 1 and 2), indicating that it has a structure other than that from N-glycosylation of the mature protein. Incubation with neuraminidase and *O*-glycanase also did not alter the 18.5-kDa protein and the nature of its structure is still unknown (Figure 2, lane 7). Since these studies were completed, Csach and Beutler have reported an 18.5-kDa secreted species of cachectin/TNF that carries a 10 amino acid N-terminal extension corresponding exactly to the propeptide sequence, apparently the result of alternate prohormone pro-

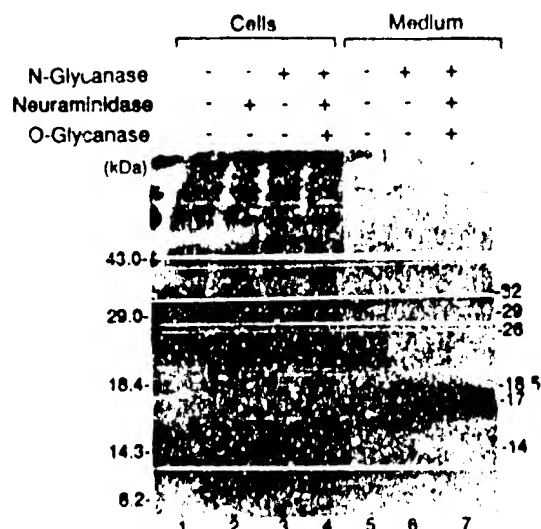


FIGURE 2: Enzymatic deglycosylation of higher molecular weight precursor and mature cachectin/TNFs. [ $^{35}$ S]Methionine-labeled cell lysates (lanes 1-4) and culture media (lanes 5-7) from LPS-stimulated RAW 264.7 cells were immunoprecipitated with antiprecachectin/TNF and anti-mature cachectin/TNF antisera, respectively. Immunoprecipitates were treated with digestion buffer alone (lanes 1 and 5), neuraminidase (lane 2), *N*-glycanase (lanes 3 and 6), or neuraminidase, *O*-glycanase, and *N*-glycanase (lanes 4 and 7) and were analyzed by SDS-PAGE and fluorography. The positions of molecular weight markers are shown on the left, and cachectin/TNF immunoreactive bands are identified by their apparent molecular weights on the right.

cessing (Csach & Beutler, 1989).

In lysates of endotoxin-stimulated RAW 264.7 cells, a 26-kDa protein was immunoprecipitated either by antibodies against prosequence or antibodies against the 17-kDa mature form of cachectin/TNF (Figure 1). Considering its apparent molecular weight, its reactivity with both antibodies, and its resistance to tunicamycin treatment, we propose that the 26-kDa protein represents the nonglycosylated 215 amino acid prohormone precursor of mature 156 amino acid 17-kDa cachectin/TNF. We also detected two bands of higher molecular mass, 29 and 32 kDa, which were also precipitated by either antiprecachectin or anti-mature cachectin/TNF antibodies (Figure 1, lanes 7 and 11). Synthesis of 29- and 32-kDa species was inhibited by treatment of cells with tunicamycin, and the two bands were no longer detectable in samples digested with *N*-glycanase (Figure 2, lanes 3 and 4). The results suggested that they were N-glycosylated forms of the 26-kDa prohormone. In a sample treated with neuraminidase, the 32-kDa band disappeared and another protein of slightly higher mobility on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was observed (Figure 2, lane 2). Treatment of the immunoprecipitate with *O*-glycanase (alone or along with other glycosidases) had no effect on the mobilities of cachectin/TNF-related proteins on gel electrophoresis (Figure 2 and data not shown). Densitometric measurement of intensities of the bands shown in Figure 1 indicated that, as with secreted cachectin/TNF, about half of the precursors were glycosylated (data not shown). The 29-kDa glycosylated precursor was present in the lysate of RAW 264.7 cells that were labeled for 2.5 min (Figure 3a), suggesting that glycosylation occurs cotranslationally.

**Proteolytic Cleavage of Precursor Cachectin/TNF.** As shown in Figure 1 (lanes 11 and 12), a broad band of protein centered at about 14 kDa was precipitated from RAW 264.7 cell lysates by antiprecachectin antibodies. This band



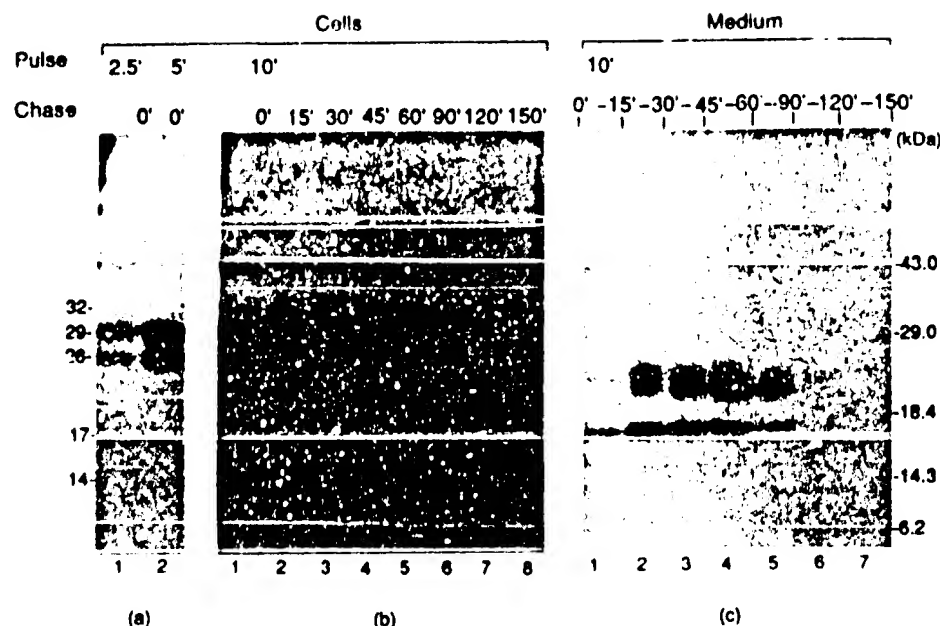


FIGURE 3: Pulse-chase analysis of the processing of the cachectin/TNF precursor. (a) RAW 264.7 cells were labeled with [ $^{35}$ S]methionine for 2.5 (lane 1) or 5 min (lane 2), washed with D-PBS and then lysed. The lysates were immunoprecipitated with antiprocachectin/TNF antisera. (b) Cells were pulse-labeled for 10 min and subsequently chased with a medium containing cold methionine. The cell lysates were prepared at the indicated times after the labeling and immunoprecipitated with antiprocachectin/TNF antisera. (c) Culture media of the cells in (b) were collected at the indicated times and immunoprecipitated with anti-mature cachectin/TNF antisera. Medium from each culture was replaced with fresh medium at the indicated times. Note, then, that time points during the chase of pulse-labeled cell lysates show the differential accumulation of radiolabel in distinct electrophoretic pools of cachectin/TNF from  $t = 0$  until the indicated time point (i.e., 0-15 min, 0-30 min, 0-45 min, etc.), whereas time points during the chase of pulse-labeled culture media show the differential proportioning of radiolabel into distinct electrophoretic pools of secreted cachectin/TNF from one time point to the next (i.e., 0-15 min, 15-30 min, 30-45 min, etc.). All samples were analyzed by SDS-PAGE; the positions of molecular weight markers are indicated on the right, and cachectin/TNF immunoreactive bands are identified by their apparent molecular weights on the left.

was precipitated only from lysates of LPS-stimulated cells and did not react with antibodies to mature cachectin/TNF. These results suggest that the 14-kDa band may represent the prosequence peptide cleaved from its cachectin/TNF prohormone precursor. Treatment of cells with tunicamycin or incubation of immunoprecipitates with *N*-glycanase, neuraminidase, and *O*-glycanase did not alter the intensity or mobility of the 14-kDa band in SDS-PAGE (Figures 1 and 2). Relative to the large amounts of mature 17-kDa cachectin/TNF released into the culture medium, only small amounts of mature 17-kDa cachectin/TNF could be detected in the cell lysate (Figure 1, lanes 7 and 8).

To test whether the 17- and 14-kDa bands might be derived from a common 26-kDa precursor, LPS-treated cells were briefly pulsed with [ $^{35}$ S]methionine and then chased for various times with cold methionine. Culture media and cell lysates were harvested at intervals up to 150 min and were analyzed by immunoprecipitation with antibodies to mature 156 amino acid cachectin/TNF or to synthetic prosequence fragments (Figure 3). Twenty-six- and twenty-nine-kilodalton proteins were precipitated by antiprotease antibodies at 10 min of pulse. Another protein of even higher molecular mass (32 kDa) appeared after 15 min of chase and the relative proportions of cachectin/TNF-like proteins above 26 kDa seemed to change gradually over time: at 60 min a 30-kDa species became a predominant protein among the 29-32-kDa proteins. As described above, these proteins are likely to represent *N*-glycosylated prohormone species of cachectin/TNF undergoing further processing in their carbohydrate groups. These proteins and the "nonglycosylated" 26-kDa precursor decreased gradually with longer chase times and had virtually disappeared after 90 min. A 14-kDa band could be detected

in the cell lysate at 10 min of pulse, increased to maximal level at 45 and 60 min of chase, and maintained a similar level for up to 2.5 h (Figure 3b). In similar experiments with a longer chase period, the 14-kDa protein disappeared completely after 3 h (data not shown).

Figure 3c shows the variety of murine cachectin/TNF species secreted into the culture medium at various times after a 10-min [ $^{35}$ S]methionine pulse. Culture medium of each dish was harvested and replaced with fresh medium at each time point to determine the amount of cachectin/TNF produced between indicated time points. When the samples were precipitated by anticachectin/TNF antibodies, mature cachectin/TNF (17 kDa) and glycosylated "ladder" proteins were detected at the first time point (0-15 min). Maximal secretion occurred from 15 to 60 min and amounts of secreted cachectin/TNF decreased thereafter. After 90 min no more labeled cachectin/TNF was secreted into the medium, which temporally coincided with the depletion of pulse-labeled cellular cachectin/TNF precursors (Figure 3b). At 15 min of chase, the nonglycosylated 17-kDa protein was the predominant species among secreted cachectin/TNFs. During later stages of the chase, glycosylated forms became more prevalent in cells and medium. The formation of the 14-kDa protein in the cell lysate was also temporally coincident with formation of mature cachectin/TNFs and the results suggest that these proteins are derived from the 26-kDa and higher molecular mass glycosylated precursors.

**Association of Precursors and the 14-kDa Prosequence of Cachectin/TNF with the Membrane/Particulate Fraction.** To localize various cachectin/TNF-related proteins in the cell, [ $^{35}$ S]methionine-labeled RAW 264.7 cells were homogenized in hypotonic buffer and the cytosol and membrane fractions

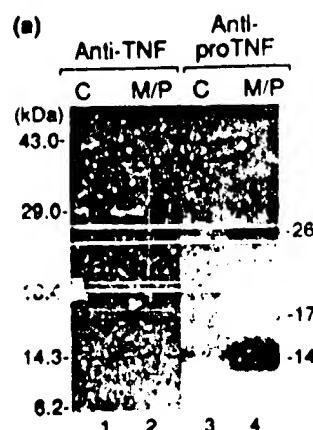


FIGURE 4: Immunoprecipitation analysis of cellular fractions. RAW 264.7 cells were stimulated with LPS and labeled with [ $^{35}$ S]methionine for 30 min. The cells were homogenized in hypotonic buffer and centrifuged to provide cytosol (lanes 1 and 3) and membrane/particulate (lanes 2 and 4) fractions. Samples were immunoprecipitated with anti-mature cachectin/TNF (lanes 1 and 2) or anti-procachectin/TNF (lanes 3 and 4) antisera and analyzed on SDS-PAGE.

were separated by centrifugation. Each fraction was analyzed by immunoprecipitation with antisera either against mature cachectin/TNF or against synthetic fragments of propeptide sequence. As shown in Figure 4, the majority of the 26-kDa precursor, N-glycosylated precursors, and 14-kDa cleaved propeptide were associated with the membrane/particulate fraction, while relatively small amounts of 17-kDa mature cachectin/TNF were present in both fractions.

To investigate the time course of appearance of cachectin/TNF as membrane-bound precursors and in soluble, mature forms, we measured cytotoxic activities of macrophage cells fixed with paraformaldehyde and of their corresponding culture supernatants. RAW 264.7 cells were incubated in medium containing LPS for up to 16 h and the cells and their culture media were harvested at various times. Paraformaldehyde-fixed cells or harvested media were then added to L-929 cells to measure cytotoxicity. Cell-associated cachectin/TNF activity could be detected 1 h after LPS addition (Figure 5). The levels of activity varied at different times but remained relatively constant: 240, 85, 650, 240, and 860 units per  $8 \times 10^4$  macrophage cells at 1, 2, 4, 8, and 16 h, respectively. The secreted cachectin/TNF increased continuously to 21200 units per  $8 \times 10^4$  cells at 16 h. The proportions of cytotoxicity associated with cells were 18%, 7%, 17%, 2%, and 4% of total cachectin/TNF activity detectable in fixed cells and culture medium at 1, 2, 4, 8, and 16 h of incubation, respectively. When culture medium containing cachectin/TNF was treated with paraformaldehyde and then dialyzed against D-PBS, no alteration in its cytotoxic activity was observed (data not shown). Release of cachectin/TNF from the fixed cells during the cytotoxicity assay period was negligible.

#### DISCUSSION

When RAW 264.7 macrophages are stimulated with LPS, they secrete a large amount of cachectin/TNF into the medium. The results shown in Figures 1 and 3 suggest that in these cells cachectin/TNF was first synthesized as a 26-kDa precursor. A similar 26-kDa protein was obtained by cell-free translation of human cachectin/TNF cDNA (Muller et al., 1986) and observed in stimulated human monocytes and NIH 3T3 cells transfected with human cachectin/TNF cDNA construct (Kriegler et al., 1988). About half of the precursor

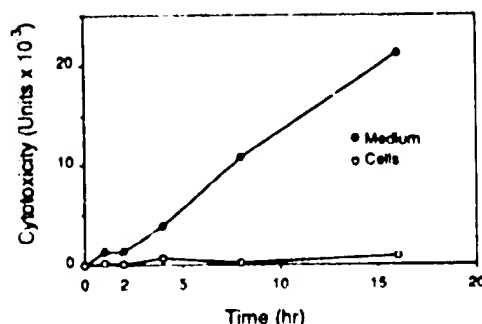


FIGURE 5: Production of cell-associated and supernatant cachectin/TNFs by stimulated RAW 264.7 cells. Cells at a density of ( $8 \times 10^4$ )/well were incubated with LPS (1  $\mu$ g/mL) for indicated times. After harvesting the culture media, cells were washed with D-PBS and fixed with paraformaldehyde. The cytotoxic activities of culture supernatant and fixed cells were measured with L-929 cells as target cells in the presence of actinomycin D.

proteins were N-glycosylated upon synthesis and migrated to the 29-kDa position on SDS-PAGE (Figure 3). Some of the N-glycosylated oligosaccharide of precursor cachectin/TNF underwent further modification before secretion. In the pulse-labeled cells, a 32-kDa protein appeared at 15 min of chase and the predominant glycosylated precursor at a later stage of processing had a molecular weight of about 30000. Results from a separate experiment showed that among the secreted "ladder" proteins only the 20-kDa protein, which has a molecular size corresponding to the cleavage product of the 29-kDa precursor protein, had significant affinity for concanavalin A (unpublished observation). This suggests that, like other secretory proteins, the 29-kDa cachectin/TNF precursor has a high mannose N-linked oligosaccharide, which is then modified to a complex type in the 32-kDa protein, containing galactose, sialic acid, and fucose (Kornfeld & Kornfeld, 1985). In fact, neuraminidase treatment reduced the apparent molecular weight of the 32-kDa protein (Figure 2). Such modifications have been reported to occur in rough endoplasmic reticulum and the Golgi complex (Kornfeld & Kornfeld, 1985).

There were at least seven or eight proteins in the "ladder" when the conditioned media of RAW 264.7 cells were analyzed by immunoprecipitation and one-dimensional gel electrophoresis, including one that migrates to the position of the primary glycosylation product (20 kDa) (Figure 1). Nonglycosylated cachectin/TNF was secreted along with the glycosylated forms and tunicamycin treatment did not affect the total amount of secreted cachectin/TNF (Figure 1; B. Sherry, D.-M. Jue, A. Zentella, and A. Cerami, unpublished results). Therefore it seems unlikely that glycosylation of cachectin/TNF greatly affects the secretion of this protein from activated macrophages. Similar findings have been reported in the secretion of serum proteins from tunicamycin-treated hepatocytes (Struck et al., 1978) and viral protein from cells infected with vesicular stomatitis virus (Gibson et al., 1979). However, it was also noted that in pulse-labeled cells the majority of secreted cachectin/TNF was nonglycosylated very early in the chase, while glycosylated cachectin/TNFs were released at later times (Figure 3c). This could mean that nonglycosylated cachectin/TNF is cleaved and released faster than glycosylated forms. Since the ratio of glycosylated to nonglycosylated cachectin/TNFs does not seem to vary greatly between newly synthesized precursor and secreted mature cachectin/TNF, posttranslational conversion of nonglycosylated prohormone into the glycosylated form seems to occur minimally, if at all.

A 14-kDa protein was found only in immunoprecipitations

of cell lysates of stimulated macrophages with antibodies to cachectin/TNF prosequence and not in immunoprecipitations with anti-mature cachectin/TNF antibodies (Figure 1). We postulate that this 14-kDa protein is the prosequence of cachectin/TNF remaining after cleavage of the mature 156 amino acid protein from the 235 amino acid precursor. The results from pulse-chase experiments further supported this suggestion (Figure 3). The molecular weight of mouse cachectin/TNF propeptides is 8660, when calculated from its amino acid sequence. Although it is not clear why the calculated (8660) and apparent molecular weights (14000) of prosequence differ to such a degree, the diffuseness of the 14-kDa band in gel electrophoresis suggests the possibility of differential posttranslational modification. However, treatment with various glycosidases did not change the pattern or mobility of the diffuse 14-kDa band (Figure 2). The possibility of posttranslational modification other than glycosylation, e.g., acylation with palmitate or myristate (Wold, 1986), should be considered.

It has been suggested that the cell surface cachectin/TNF precursor plays a role in cell-cell communication and in confining the action of cachectin/TNF to local tissues that are in close contact with activated macrophages (Decker et al., 1987; Kriegler et al., 1988). When we measured cell surface cachectin/TNF by adding paraformaldehyde-fixed RAW 264.7 cells to L-929 cells, a substantial amount of cytotoxic activity could be detected in cells stimulated with LPS for 1 h (Figure 5). However, there was no further accumulation of cytotoxic activity, which persisted at a relatively low level up to 16 h, while the level of secreted cachectin/TNF increased continuously. This result suggests that in activated macrophages membrane-bound cachectin/TNF precursor is an intermediate in the generation of mature cachectin/TNF. In fact, when we measured the band intensities of the proteins shown in Figure 1 by densitometry, the 26-kDa and glycosylated precursors constituted 14% of the total cachectin/TNF-related proteins (data not shown). If it is assumed that macrophages actively produce cachectin/TNF for 6 h and the amount of precursors displayed on the cell surface remains constant during this period, it can be estimated that at most 1% of produced cachectin/TNF is present as membrane-bound precursor at any point in time. However, it is obvious that a certain amount of cachectin/TNF precursor is present on the surface of activated macrophages and it might be used in cell-cell interactions.

Kriegler et al. (1988) suggested that cachectin/TNF precursor is transported to the cell surface and then cleaved by a proteolytic enzyme outside the cell. Our result with paraformaldehyde-fixed macrophages also showed the presence of cachectin/TNF on the surface of activated cells (Figure 5). However, it is not certain whether the plasma membrane is the only site for the release of cachectin/TNF or if the cleavage occurs also in other intracellular organelles, such as the endoplasmic reticulum or the Golgi apparatus. The presence of the mature form of cachectin/TNF in the cell lysate might suggest that the precursor is also cleaved inside the cell (Figures 1 and 4). Cachectin/TNF produced in these organelles might then be secreted like other secretory proteins. The intracellular 17-kDa mature protein does not seem to come from receptor-mediated uptake of secreted cachectin/TNF, since we could not see any detectable amount of radiolabeled 17-kDa cachectin/TNF in the lysate of macrophages that were incubated with [<sup>35</sup>S]methionine-labeled mature cachectin/TNF (unpublished observation). It is possible, however, that the mature protein is generated by endogenous protease during

lysis of cells, although we added several protease inhibitors with different specificities to the lysis buffer.

Previous reports (Muller et al., 1986; Decker et al., 1987; Kriegler et al., 1988) and our results show that cachectin/TNF is produced as a membrane-associated precursor that is then proteolytically cleaved to release mature cytokine into the medium. Recently, a number of cytokines produced by macrophages (IL-1) (Kurt-Jones et al., 1985; Matsushima et al., 1986), tumor cells (transforming growth factor  $\alpha$ ) (Bringingman et al., 1987; Teixeira et al., 1987) and mesenchymal cells (colony stimulating factor 1) (Rettenmier et al., 1987; Rettenmier & Roussel, 1988) have been shown or suggested to be secreted by similar mechanisms. Although the biological significance of membrane-bound precursors of these cytokines has not yet been clarified, it is obvious that the mode of secretion differs from that common to other secretory proteins. The cleavage of the signal peptide of other secretory proteins by signal peptidase is known to occur cotranslationally during the translocation of the nascent polypeptide chain across the endoplasmic reticulum (Blobel et al., 1979). The difference suggests that these cytokines are secreted by mechanisms that include proteolysis by enzymes distinctive from the well-known signal peptidase. By characterizing the enzymes involved, it should be possible to understand the mechanism and significance of this unusual mode of protein secretion.

#### ACKNOWLEDGMENTS

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## Estrogen Receptor Binding to a DNA Response Element in Vitro Is Not Dependent upon Estradiol<sup>†</sup>

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**ABSTRACT:** Gel shift assays were employed to distinguish between the contribution of 17 $\beta$ -estradiol (E<sub>2</sub>) and a short heating step to the ability of the rat uterine cytosolic estrogen receptor (ER) to bind to the estrogen response element (ERE) from the vitellogenin A2 gene (vitERE). Despite the popularity of models in which the ER is a ligand-activated DNA-binding protein, these studies find that estrogen does not significantly contribute to receptor-DNA complex formation. An avidin-biotin complex with DNA (ABCD) assay was utilized to obtain quantitative measurement of the affinities of the ER for the vitERE and a mutant sequence. Scatchard analysis gave a dissociation constant of 390  $\pm$  40 pM for the E<sub>2</sub>-occupied, heated ER to the vitERE. The data fit a one-site model and evidence for cooperativity was not observed. A dissociation constant of 450  $\pm$  170 pM was obtained for the unoccupied, heated ER, leading to the conclusion that estrogen was not necessary for specific binding to DNA. The percentage of ER capable of binding vitERE varied with each cytosol preparation, ranging from 60 to 100% and estrogen did not appear to affect this variation. Competition against the vitERE with a 2-bp mutant sequence showed a 250-fold lower relative binding affinity of the receptor for the mutant over the vitERE sequence. This ability of the ER to discriminate between target and nonspecific DNA sequences was also not dependent on the presence of estrogen.

It has been widely proposed that estrogens effect gene transcription by inducing their receptor protein to bind to specific DNA sequences in target genes. This attractive model of steroid receptors as ligand-activated DNA-binding proteins has been commonly presented as established consensus in the literature (Baulieu, 1989; Evans, 1988; Hunt, 1989; Picard et al., 1988, 1990) and even in a recent textbook (Alberts et al., 1989). This model traces its origin to the fact that a characteristic of the in vitro transformed estrogen receptor

(ER) is its ability to bind to DNA-cellulose, albeit with only micromolar affinity and a modest increase in affinity induced by steroid (Skafar & Notides, 1985). A number of treatments, including heating or high salt, lead to receptor transformation and this process was generally agreed to also require the presence of hormone (Grody et al., 1982). The receptor would be expected to display a 1000- to 10000-fold increase in binding affinity to target DNA sequences in response to hormone according to theoretical calculations, if, indeed, hormone confers the ability of the receptor to select target sequences over the mass of DNA in the nucleus (Lin & Riggs, 1975; Ptashne, 1984; Travers, 1983, 1984; von Hippel & Berg, 1989).

One such target sequence for estrogen action has been identified in the 5'-flanking region of the vitellogenin genes of *Xenopus* and chicken (Klein-Hitpass et al., 1986; Walker et al., 1984). Transcription of these genes in vivo is strongly dependent on the presence of estrogen (Wahli, 1981). The

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# **STIC-ILL**

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**Sent:** Monday, August 30, 1999 2:19 PM  
**To:** STIC-ILL  
**Subject:** 08/981,559 Van Ostade X; Tavernier J; \*\*\*Fiers W\*\*\*

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Comp. of wt and mut TNF



## REVIEW

# Structure-activity studies of human tumour necrosis factors

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The mechanism by which tumour necrosis factors (TNF and lymphotoxin, also called TNF $\alpha$  and TNF $\beta$  respectively) exert their cytotoxic activity on many malignant cells, remains largely unknown. Furthermore, the broad array of differentiation (gene induction) and mitogenic activities towards many primary cells is still a subject of intensive investigation. TNF is an important mediator in inflammation, immune responses and infection-related phenomena and these activities contribute to the severe toxicity seen when TNF is used as an anticancer agent. The first step in the mechanism of action is the specific binding of the ligand to its receptors and dissection of the molecular mechanism involved in this interaction is the subject of this review. The reasons for the interest in this aspect are obvious: first, the development of strong antagonistic TNF analogues can be useful in dampening the potentially lethal or debilitating effects of an overproduction of the cytokine (as in septic shock or rheumatoid arthritis). Secondly, since two distinct TNF receptors exist, construction of TNF mutants that distinguish between both types may lead to derivatives of this pleiotropic agent with a more restricted biological activity pattern. Ideally, one would like to develop a TNF mutant that has retained its cytotoxic action on tumour cells without inducing the deleterious systemic toxicity. Such an optimized TNF molecule could become a potent anticancer agent.

**Key words:** active site/lymphotoxin/mutagenesis/structure-function/tumour necrosis factor

## Primary structures

Since the cloning of human and mouse tumour necrosis factor (TNF) and lymphotoxin, some 8 years ago (Gray *et al.*, 1984, 1987; Pennica *et al.*, 1984, 1985; Fransen *et al.*, 1985; Marmenout *et al.*, 1985), the genes for rabbit (Ito *et al.*, 1986), rat (Shirai *et al.*, 1985), cat (McGraw *et al.*, 1990), dog (Fiers, 1993), porcine (Pauli *et al.*, 1989; Drews *et al.*, 1990), goat (Sprang and Eck, 1992), bovine (Niitsu and Watanabe, 1988), equine (Su *et al.*, 1991) and ovine (Young *et al.*, 1990; Green and Sargan, 1991) TNF and for rabbit (Ito *et al.*, 1986) and bovine (Niitsu and Watanabe, 1988) lymphotoxin have been isolated by different groups (Figure 1). Conservation within the TNF and lymphotoxin sequences is 71 and 61% respectively and comparison between the two human cytokines reveals an identity of 32%, when the alignment was optimized, based on the comparison of their 3-D structures (Eck *et al.*, 1992; see below). Cloning of the cDNA for the human and mouse membrane-bound

T-cell antigen CD40L (Armitage *et al.*, 1992; Hollenbaugh *et al.*, 1992) revealed an identity of 22% when the extracellular domain of human CD40L was aligned with the human TNF and human lymphotoxin amino acid sequences (Figure 1). The corresponding receptor for this ligand is the B-cell CD40 antigen, a member of a family of transmembrane proteins, including the low-affinity nerve growth factor (NGF) receptor and the two different TNF receptors, TNF-R55 and TNF-R75 (see below). Recently, a fourth member of the TNF family, called p33 or lymphotoxin- $\beta$ , was identified and found to be 23 and 27% identical to TNF and lymphotoxin respectively (Figure 1; Browning *et al.*, 1993). p33 forms a heteromeric complex with lymphotoxin at the cell surface (see below).

Asparagine-linked glycosylation was demonstrated on murine TNF and on murine and human lymphotoxin, at positions 7, 60 and 62 respectively (Haranaka *et al.*, 1986; Porter, 1990), but not on human TNF (potential *N*-glycosylation sites can also be found in several other species). Moreover, it was shown that *O*-glycosylation on Thr7 could contribute to the heterogeneous molecular mass forms of natural human lymphotoxin preparations (Voigt *et al.*, 1992). Also CD40L is supposed to be *N*-glycosylated since its calculated  $M_r$  (29 395 Da) does not correspond to its observed  $M_r$  (32–33 000 Da) (Armitage *et al.*, 1992; Hollenbaugh *et al.*, 1992). Except when indicated in this report, positions in the protein will be numbered, starting from Val1 of human TNF. Negative numbering stands for residues in the pre-sequence with Ala1 being the first residue upstream of Val1.

## Tertiary structure

X-ray diffraction data on TNF (Eck and Sprang, 1989; Jones *et al.*, 1989, 1990a, 1991; Sprang and Eck, 1990, 1992) and lymphotoxin crystals (Eck *et al.*, 1992) reveal the 3-D structures of both cytokines (Figure 2a and b). Each subunit, folded into a 'jelly-roll' antiparallel  $\beta$ -sheet sandwich, forms a bell-shaped, rigid, trimeric molecule with two other subunits. As a result, a channel is formed along the trimer axis, lined by charged and polar residues near the top and bottom and by hydrophobic residues in the middle part. Conserved residues between TNF and lymphotoxin cluster predominantly in the inner core of the molecule, when a slightly different alignment was used also (Tavernier *et al.*, 1989). As expected, the exterior surface is composed mostly of charged and polar residues. The monomers show a striking structural homology with some viral capsid proteins such as influenza haemagglutinin and especially satellite tobacco necrosis virus (STNV) coat protein. The only cysteine bridge in each TNF monomer is localized near the top, while the N- and C-termini are located at the bottom of the structure. Dimers or trimers have been reported (Sprang and Eck, 1990), but it remains to be established whether such a higher order quaternary structure represents a biologically relevant entity rather than a crystallographic curiosity.

Since the regions of homology between CD40L or p33 and TNF/lymphotoxin fall within the  $\beta$ -sandwich forming sequences (Figure 1), a similar 3-D fold has been suggested for CD40L

**Fig. 1.** Primary sequence of different mammalian TNF, lymphotoxin, CD40L and p33 species (see text for references). Conserved TNF, conserved LT and conserved CD40L stand for identical residues in the different TNF (one exception is accepted), lymphotoxin and CD40L species respectively. Conservation between human TNF and human lymphotoxin, cons. hTNF-hLT; between human CD40L and human TNF, cons. hCD40L-hTNF; between human CD40L and human lymphotoxin, cons. hCD40L-hLT; between human p33 and human TNF, cons. hp33-hTNF, between human p33 and human lymphotoxin, cons. hp33-hLT. Small letters indicate that the conservation in one of the structures is not fully conserved between the different species (for TNF, one exception is accepted). Residues involved in secondary structure of TNF and lymphotoxin (line 14) are indicated as i and o (= inside and outside orientation in the monomer  $\beta$ -sandwich respectively).  $\beta$ -Strands on the sheet: facing the trimer axes are underlined (adapted from Srour and Eck, 1992).

(Farrah and Smith, 1992) and p33 (Browning *et al.*, 1993). Hence, it can be proposed that, in analogy with the hypothesis of Bazan (1990) for the cytokine receptor family, the ligands of the TNF/NGF receptor family also share some structural (and even sequential) homology. However, although NGF is also an all- $\beta$  protein, its topology is complete, different from TNF and lymphotoxin (McDonald *et al.*, 1991) and the TNF/NGF ligand family could thus fall into two structural classes (Farrah and Smith, 1992).

Human TNF is synthesized as a precursor protein of 233 amino acids. The prosequence has an unusual length of 76 amino acids and is very conserved in all species of the TNF family (76%), suggesting an as yet unknown but important biological role. In this prosequence, a hydrophobic stretch between residues -44 and -26 is present (Fiers *et al.*, 1991b). Since it was shown that TNF can also function as a surface-bound cytotoxin (Decker *et al.*, 1987; Liu *et al.*, 1989), this stretch could serve as a

membrane anchor of the TNF prohormone, as has been demonstrated by several groups (Kriegler *et al.*, 1988; Luetig *et al.*, 1989; Kinkhabwala *et al.*, 1990). The myristyl acylation of a couple of lysine residues, upstream of this sequence, may facilitate the membrane insertion process (Stevenson *et al.*, 1992). Pro-TNF should thus have a type II membrane glycoprotein configuration since its C-terminus is located extracellularly and this characteristic is shared with the CD40L ligand (Armitage *et al.*, 1992; Hollenbaugh *et al.*, 1992).

The unusual processing of this precursor molecule is remarkable, since no release of the 17 kDa mature TNF subunit was detected in a cell-free system, supplemented with dog pancreas microsomes, but processing to TNF polypeptides of various lengths (17, 18.5 and 20 kDa) was observed in mammalian cells and *Xenopus laevis* oocytes (Müller *et al.*, 1986). It has been suggested that the TNF precursor is transported to the cell surface and then cleaved by extracellular protease activity, thereby leaving behind a 14 kDa prosequence in the cell membrane (Jue *et al.*, 1992). The difference in calculated (8660 Da) and apparent (14 kDa)  $M_r$  and the absence of glycosylation, supports the idea of other post-translational modifications of the membrane-inserted prosequence, such as myristyl acylation (Jue *et al.*, 1992). An 18.5 kDa mature subunit was also characterized in the medium upon induction of the mouse RAW264.7 cell line and it was shown that this variant started with ten additional N-terminal amino acids that rendered the molecule biologically inactive (Cseh and Beutler, 1989). In this respect, it may be mentioned that the *in vitro* translated precursor TNF is also inactive (Kriegler *et al.*, 1988), suggesting that segments of the prosequence can mask the active centre or constrain the molecule into an inactive form. Because cleavage within the region between amino acids -32 and -1 should result in the 17, 18.5 and 20 kDa forms, Klostergaard *et al.* (1992) deleted this sequence and replaced it by the tetrapeptide Ile-Asp-Leu-Glu, but unexpectedly no uncleavable forms could be found. A similar result was observed when small deletions within the -3, +5 region were introduced (Perez *et al.*, 1990). A deletion of the first 12 amino acids disrupts the cleavage process of TNF and leaves a membrane-bound, active form (Perez *et al.*, 1990).

The signal sequence of lymphotoxin (34 amino acids) is more standard and does not allow membrane anchoring. Nevertheless, membrane-bound lymphotoxin was detected (Abe *et al.*, 1991; Hiserodt *et al.*, 1992). Here, anchoring occurs by the non-covalent interaction of one lymphotoxin monomer with a membrane-bound dimer of a p33 protein (Browning *et al.*, 1991; Androlewicz *et al.*, 1992). Further studies must unravel whether this lymphotoxin-p33 heterotrimer exerts activity and, if so, how far it mimics that of lymphotoxin. Certainly, this capacity of the lymphotoxin monomer to combine with other molecules is, in view of its structure-function relationship, an intriguing phenomenon that could shed new light on the mechanism of action of lymphotoxin.

#### Physicochemical characteristics

A summary of the biochemical and biophysical properties of TNF and lymphotoxin is given in Table I. Many attempts have been made to determine the mol. wt of native TNF and lymphotoxin by gel filtration, but specific interactions with the different column matrices (Yoshimura *et al.*, 1980; Petersen *et al.*, 1989; Schoenfeld *et al.*, 1991) were at the source of confusing conclusions. Moreover, conflicting reports have been published, concerning the amount of monomeric TNF in solution (Narhi

and Arakawa, 1987; Smith and Baglioni, 1987; Schoenfeld *et al.*, 1991; Corti *et al.*, 1992). However, methods such as ultracentrifugation and sedimentation, cross-linking, small angle light scattering and 2-D gel electrophoresis showed that TNF under all physiological circumstances is in a trimer form (Table I).

On the whole, TNF is relatively resistant to organic solvents and acidic conditions as compared with lymphotoxin (Aggarwal, 1990; Porter, 1990). The side chain of Lys98 in TNF forms an ionic bond with the carboxylate group of Glu116 from the same subunit and this results in a stabilizing ring of ion pairs around the 3-fold axis near the top of the molecule. Since Glu116 is replaced in lymphotoxin by a histidine residue, the lack of charge compensation may account for the acid lability of the molecule (Eck *et al.*, 1992). In contrast, lymphotoxin is more resistant to protease activity. Indeed, several highly accessible protease recognition sites are exposed near the bottom of the TNF molecule, where the putative active site is located (see below), while in lymphotoxin, these sequences map near the less sensitive N-terminal part or the upper region of the molecule (Jones *et al.*, 1991).

It should be noted that, although unglycosylated lymphotoxin has the same bioactivity as the glycosylated natural product, the presence of the carbohydrate group renders the molecule more hydrophilic (Hains and Aggarwal, 1989) and makes it more accessible for iodination (Stauber and Aggarwal, 1989).

The modification of TNF with reagents specific for the imidazole moiety of histidine (Yamamoto *et al.*, 1987, 1989) or for the free amino groups of lysine and the N-terminal valine (Klostergaard *et al.*, 1992; Utsugi *et al.*, 1991, 1992), resulted in a decrease of cytotoxicity, correlating with the degree of TNF modification. This led in the former case to the identification of His15 as a possible candidate for participation in the active site (see below). On the other hand, acylation of some amino groups can lead to an increase in hydrophobicity without loss of TNF activity and such a modified TNF molecule can integrate more easily into lipid vesicles. It has been claimed that lipid-bound TNF possesses unchanged *in vitro* tumor cytotoxicity and immunomodulatory effects, while some toxic side-effects *in vivo* would be reduced (Debs *et al.*, 1989, 1990).

#### Determination of the active site

Mutational analyses of TNF have been facilitated by the high TNF expression levels that could be reached in *Escherichia coli* cells. Furthermore, the fact that TNF does not form inclusion bodies but remains soluble in bacterial extracts, allows a fast and easy purification for wild type TNF as well as for many of the mutants. Lymphotoxin is much less soluble in *E. coli* lysates. However, efficient purification protocols (Schoenfeld *et al.*, 1991), together with optimization of the ribosome binding site and adapted codons for *E. coli* throughout the gene (Seow *et al.*, 1989), have allowed lymphotoxin to be obtained in high quantities from bacteria.

The TNF and lymphotoxin mutants published so far are summarized in Table II. Many of the substituted residues are located in the interior of the monomeric and/or trimeric molecule. Not unexpectedly, any change in these usually highly conserved amino acids induces a structural distortion that indirectly causes inactivity. Drastic conformational disturbances can be detected by changes in solubility or immunoreactivity. Also, several techniques such as gel filtration, refolding behaviour, CD (circular dichroism), cross-linking, fluorescence and a combination of IR (infrared spectroscopy) with hydrogen exchange, have been used to evaluate the global conformation



Table I. Physicochemical characteristics of TNF and lymphotoxin

	TNF		LYMPHOTOXIN	
number of amino acids	human : 157 mouse : 156		human : 171 mouse : 169	
cysteine cysteine-bridge	human : Cys69, Cys101 mouse : Cys69, Cys101 C69-C101	Davis et al., 1987 Hsu et al., 1986 Wingfield et al., 1987	human : - mouse : Cys84	
N-glycosylation	human : - mouse : present(Asn7)	Green et al., 1976 Harasaka et al., 1986 Jue et al., 1990	human : present(Asn62) mouse : present(Asn60)	Aggarwal et al., 1985b Porter et al., 1990
O-glycosylation			human : present(Thr7)	Voigt et al., 1993
molecular weight (Da) : theoretical	17356 (monomer) 52068 (trimer) human : 17000	Pennica et al., 1984	18664 (monomer) 55992 (trimer) human : 18800-25000	Aggarwal et al., 1984 Gray et al., 1984 Hains et al., 1989 Seow et al., 1989 Porter et al., 1990 Wakabayashi et al., 1990
SDS-PAGE	mouse : 15-18000	Kull and Cuatrecasas, 1984 Pennica et al., 1985 Harasaka et al., 1986		Browning and Ribolini, 1989
cross-linking	±43000	Browning and Ribolini, 1989 Lam et al., 1988 Smith and Baglioni, 1987 Eck et al., 1988	±50000	
2D-ODD-IEF <sup>a</sup>	55000 trimer composed of three slightly different subunits	Schoenfeld et al., 1991 Wingfield et al., 1987 Sreekrishna et al., 1989 Smith and Baglioni, 1987 Arakawa and Yphantis, 1987	56000	Schoenfeld et al., 1991
sedimentation and ultracentrifugation	49000 50000 51000 52000 54000			
small angle scattering mol. weight	48000 50-53000	Schoenfeld et al., 1991 Yoshimura et al., 1988 Lewit-Bentley et al., 1988	46000	Schoenfeld et al., 1991
crystallization wild-type	trigonal (3.5 Å) rhombohedral (1.85 Å, twinned) tetragonal (<3 Å) trigonal (3 Å) cubic (3 Å) hexagonal (unstable) trigonal (2.9 Å) trigonal (3.2 Å) rhombohedral (1.8 Å, twinned)	Lewit-Bentley et al., 1988 Fiers et al., 1986 Eck et al., 1988 Hakoshima and Tomita, 1988 Jones et al., 1989 Sahudjian et al., 1993 Van Ostade, unpubl. res.	hexagonal (1.9 Å)	Eck et al., 1992
mutant A84V C69S				
Spectra CD (circular dichroism)	near UV : max : 280.5 nm : 285.5 nm : 291-292.5 nm  far UV min : 219-221 nm	Davis et al., 1987 Hsu et al., 1986) Naruchi et al., 1987 Wingfield et al., 1987 Van Ostade et al., 1991 Sreekrishna et al., 1989 Nishikawa et al., 1990	max : 264 nm : 269 nm : 281 nm : 289 nm : 296 nm  min : 220 nm	Goh et al., 1992 Nishikawa et al., 1990  Wakabayashi et al., 1990 Nishikawa et al., 1990
fluorescence (280 nm)	max : 319 to 331 nm quenching with $^{125}$ I, AAB <sup>b</sup>	Naruchi et al., 1987 Arakawa et al., 1990		
IR-spectroscopy in combination with H-exchange	β-structure : 35% α-helix : 9% β-turns : 22% loops : 10%	Prestrelski and Arakawa, 1991		

isoelectric point	human : 5.2-5.9  : 5.8-6.8  mouse : 3.9 : 4.6-4.8	Aggarwal et al., 1985b Yamada et al., 1985 Geigert et al., 1987 Porter et al., 1990 Eck et al., 1988 Sreekrishna et al., 1989 Haranaka et al., 1986 Aggarwal et al., 1991a, 1991 Kull and Custrone, 1981	human : 5.8  : 6.9  : 8.3	Aggarwal et al., 1984 Porter et al., 1990 Hains and Aggarwal, 1989  Schmidfield et al., 1991
reaction with: <i>diethylpyrocarbonate</i> (DEP)	concomitant activity reduction	Yamamoto et al., 1987, 1989		
<i>N-Hydroxysuccinimide</i> ester	concomitant activity reduction	Utsumi et al., 1991; 1992 Klotzgaard et al., 1992		
stability within pH region	5.5-10 4.8-10.5 and denaturation at 4.3	Haranaka et al., 1986 Wingfield et al., 1987	unstable in acidic conditions	Porter et al., 1990
detergents	relatively stable  dissociation at 1% NP-40 stable at 2% NP-40	Porter et al., 1990  Browning and Ribolini, 1989 Lam et al., 1988	unstable  stable at 1% NP-40	Porter et al., 1990  Browning and Ribolini, 1989
urea	denaturation at 3.8 M	Wingfield et al., 1987		
guanidinium-HCl	denaturation at 1.14 M	Wingfield et al., 1987		
protease sensitivity - trypsin	+	Aggarwal et al., 1985c Narhi et al., 1989 Haranaka et al., 1986 Soudri et al., 1991	±	Aggarwal et al., 1985b
- elastase	+	Bauvois et al., 1992	+	Soudri et al., 1991
- α-chymotrypsin	+	Haranaka et al., 1986 Aggarwal et al., 1985c Haranaka et al., 1986	-	Aggarwal et al., 1985b
- pepsin	±	-	-	-
- cathepsin G	+	Soudri et al., 1991 Bauvois et al., 1992	+	Soudri et al., 1991
- papain	-	Haranaka et al., 1986	-	-
- S. aureus VII	+	Aggarwal et al., 1985c	-	-
- macrophage-bound proteases :	-	-	-	-
- N-aminopeptidase	-	Bauvois et al., 1992	-	Aggarwal et al., 1991
- Ser-endopeptidase	+	-	-	-
temperature 37°C-40 hr. 56°C-6 hr. 70°C-1hr. 100°C-2 min. 25°C-1 month 25°C-4 months 37°C-1 month	stable reduced activity no activity no activity reduced activity degraded degraded	Wingfield et al., 1987 - Green et al., 1976 Wingfield et al., 1987 Geigert et al., 1987 - -		

Except when indicated, data were derived from experiments with human TNF.

<sup>a</sup>2D-ODD-IEF: two dimensional, one dimension denatured isoelectric focusing.

<sup>b</sup>AA: acrylamide

of TNF analogues. In addition, crystallization and X-ray diffraction of the inactive TNF mutant A84V (see below) is under way (Saludjian *et al.*, 1992). In the cases where loss of bioactivity was not accompanied by a gross structural alteration, the concerned residue was boxed and assumed to lie in or very near the active site (Figure 3a and b). Exceptions to this rule are residues S60 and H78 which are known to be positioned inside the molecule. Some mutations at these sites probably induce local

conformational changes, leading to biological inactivity, but which are undetectable by physicochemical assays (Table II, Zhang *et al.*, 1992). The most relevant mutations, with respect to the TNF active site, will be discussed below.

#### Importance of the N- and C-termini

Deletion at the N-terminus for eight amino acids increases the activity of TNF by a factor of 1.5-5, as measured in an L929

Table II. List of mutations in TNF and lymphotoxin

mutation	cytotox.	SDS GE	imm. react.	3D	ref.		ND	-	ND	PS	
wild-type	100	++	100			Δ150-157	ND	-	ND	PS	Lin et al., 1992
26 kDa	< 0.1		+		Kriegler et al., 1988	Δ151-157	ND	-	ND	PS	Sidhu and Bolton, 1989
					Perez et al., 1988	Δ155-156		-		PS	Gase et al., 1990; 1991
18.5 kDa	< 1.0	++			Cash and Beutler, 1989	Δ155-157		-		PS	"
wild-type+ R-I-R-M	> 100 <sup>a</sup>				Soma et al., 1987	Δ156-157	ND	-	ND	PS	Sidhu and Bolton, 1989
Δ1-7+P8R+T9D+	100		100		Kobayashi et al., 1986	Δ157	ND	-		PS	Gase et al., 1990; 1991
Δ10R+T26N						R 2 K+S4H+T7H	260				Lin et al., 1992
Δ1-7+P8R+M9D+	<10 <sup>-4</sup>					K+S4C+S5T	118	++		PS	Gase et al., 1990; 1991
Δ10R+T26N+Δ162-171						+D10R					Kamijo et al., 1989
Δ1-2	150		+		Nakamura et al., 1990	K+S4C+S5T	17	++			Soma et al., 1989
	460				Kamijo et al., 1989	+D10R+P12A					"
Δ1-2+L157F	10					K+S5T+D10R	194	++			"
Δ1-2+S3T+S4T+S5T	190					K+S5T+D10R	<17	++			"
Δ1-4	150				Creasey et al., 1987	+P12A					"
Δ1-5	150		±100		Tsujiimoto et al., 1987	S 4 C+S5T+D10R	165	++			"
Δ1-6	±190				Nakamura et al., 1990	C+S5T+D10R	<17	++			"
Δ1-25					Aggarwal et al., 1985b	+P12A					"
Δ1-7	240				Creasey et al., 1987	S 5 T+D10R	235	++			"
	275		+		Nakamura et al., 1991b	T+D10R+P12	<17	++			"
	360		±100		Sidhu and Bolton, 1989	D 10 R	40				Ito et al., 1991
Δ1-7+P8R+S9K+D10R	600	++			Van Ostade, unpubl. res.	K 11 Q	±100	++			Zhang et al., 1992
Δ1-7+R32E	0.03	+	+	GF	Nakamura et al., 1991a	M	±100	++			"
Δ1-7+R32Y (mouse)	33	++			Nakamura et al., 1991b	T	±100	++			"
Δ1-7+A35H	5				Van Ostade et al., subm.	N	±100	++			"
Δ1-7+A84H	ND		+	GF	Masegi et al., 1989	A 14 T	ND	-	0.01		Yamagishi et al., 1990
Δ1-24	86 <sup>b</sup>			CD	Nakamura et al., 1991b	H 15 Y	0.004	-	0.01		Ito et al., 1991
Δ1-24+Δ84H	10 <sup>5</sup> b			RF	Nakamura et al., 1991b		ND	-	0.01		Yamagishi et al., 1990
Δ1-8	170			RF	Wakabayashi et al., 1990		0.005	++ <sup>a</sup>		CL	Zhang et al., 1992
	400			CD			0.015	++ <sup>a</sup>		CL	"
Δ1-25	±100	++					0.0001				Yamamoto et al., 1987; 1989
Δ1-9	100	++			Creasey et al., 1987	Q	15				"
Δ1-26	100	++			Geiger et al., 1987	G	ND				"
Δ1-10	10-35				Nishikawa et al., 1990	K	ND				"
	33	++			Mark et al., 1987		ND				Van Ostade, unpubl. res.
Δ1-27	±100	++		CD	Nishikawa et al., 1990	D	0.01	++ <sup>a</sup>			Zhang et al., 1992
Δ1-27+K24Q+K29N+	4x10 <sup>-4</sup>				Carlino et al., 1987	P	0.001	++ <sup>a</sup>			"
Y95V	4.5x10				Creasey et al., 1987	V 16 A	ND	-	0.01		Yamagishi et al., 1990
Δ1-27+K29N+Y95N					Mark et al., 1987	A 18 V	2	+	30		"
Δ1-11	ND	+			Nakamura et al., 1991b	N 19 T	0.7	-	2		"
Δ1-28	ND				Wakabayashi et al., 1990	D 36 N	±100				Goh et al., 1992
Δ1-12	ND					H	±100				"
Δ1-17	ND					Y	50				Goh et al., 1991b
Δ1-17 + R-I-R-M	>wt					P 20					"
Δ1-20	ND					P 37 T	±100				Goh et al., 1992
Δ1-37	ND					A	±100				"
Δ31-35+GSG	0.0005	++			Nishikawa et al., 1990	S	±100				"
Δ137-157	-	-		PS	Nakamura et al., 1991b	Q 21 L	46	++	68		Yamagishi et al., 1990
Δ137-138+Δ140-141	-	-		PS	Nakamura et al., 1990	E 23 V	65	++	84		"
Δ142-157					Soma et al., 1987	L 26 F	0.1	-	2		"
Δ156-171	ND					Q 27 P	±100	++			Zhang et al., 1992
						L	±190	++			"
						H	±100	++			"
						K	±100	++			"
						W 28 F	45	+		GF	Van Ostade et al., 1991
						F+W114F	ND	-			Van Ostade, unpubl. res.
						R	ND	-	ND		Yamagishi et al., 1990
						C	100	++			Zhang et al., 1992

[illegible]

Shaded mutations were carried out on the corresponding positions in lymphotoxin and the resulting activities were compared with wild type lymphotoxin. SDS-GE, control of the solubility and expression behaviour of the mutants by SDS gel electrophoresis; imm. react., control of the immunoreactivity of the mutants by reaction with a monoclonal anti-TNF antibody; 3-D, control of the global 3-D structure of the mutants by various physicochemical techniques; ND, not detectable; CD, circular dichroism; RF, refolding behaviour; GF, gel filtration; PS, protease sensitivity; CL, cross-linking; FL, fluorescence; Q,

	C	0.01	++		CL	-
	S	0.03	++		CL	-
L 120	Q	7	+	21		-
V 123	G	0.02	-	0.01		-
L 126	Q	ND	-	ND		-
G 127	A	±100	++			Van Ostade, unpubl. res.
D 130	A	ND	++	0.08		Yamagishi et al., 1990
	N	ND	++	0.1		-
R 131	C	46				Ito et al., 1991
	Q	100	++	ND		Tavernier et al., 1990
Q 146	K	±100				Goh et al., 1991a
S 133	I	112	++	53		Yamagishi et al., 1990
		0.6	++		CL	Zhang et al., 1992
	C	50	++		CL	-
	G	0.1	++		CL	-
	T	0.2	++		CL	-
A 134	T	104	++	40		Yamagishi et al., 1990
E 135	K	4	++	68		-
H 136	Y	±100				Goh et al., 1991a
N 137	D	12	+	-		Yamagishi et al., 1990
R 138	L	100	++	5		Tavernier et al., 1990
	C+S147F	140				Ito et al., 1991
D 140	K	-				Tavernier et al., 1990
	Y	2	+	6		Yamagishi et al., 1990
Y 141	C	35	++	58		-
L 142	R	ND	-	0.02		-
D 143	Y	ND	++	53		-
Y 144	D	±1				Goh et al., 1992
	K	±1				-
F 144	S	38	++	74		Yamagishi et al., 1990
	Y	20	++	74		-
A 145	V	2	+	58		-
S 146	A	±100				Goh et al., 1992
E 146	K	0.0002	++		CD	Van Ostade et al., 1991
	H	0.0006	++			Van Ostade et al., subm.
	Q	70	++			-
		ND	++	32	CL	Yamagishi et al., 1990
S 147	A	100	++		CL	Zhang et al., 1992
	T	100	++		CL	-
	F	100	++		CL	-
	Y	0.01	++		CL	-
	C	0.1	++		CL	-
		0.04	+	17		Yamagishi et al., 1990
152	R+(V,N) <sub>2</sub>	ND				Kobayashi et al., 1986
G 148	E	0.009	+	7		Yamagishi et al., 1990
V 150	D	0.7	+	5		-
	G	0.5	+	6		-
F 152	L	0.2	-	0.2		-
G 153	E	ND	-	ND		-
I 155	L	14	++	48		-
L 157	F	146	++	95		-
		490				Kamijo et al., 1989
	M	77	--	42		Yamagishi et al., 1990
		130				-
	P	15	++	31		Ito et al., 1991
	Q	6	-	2		Yamagishi et al., 1990
	V	20	++	58		-

fluorescence quenching; DS, dissociation behaviour; IR, infrared spectroscopy combined with H-exchange; XR, X-ray diffraction analysis. A deletion is represented by a Δ symbol and Δ1-7 indicates that the first seven residues were removed. An asterisk points to the presence of aggregated forms, seen on SDS gels. Positions at which mutants were clearly reduced in cytotoxic activity and maintained their immunoreactivity and/or physico- or biochemical characteristics are boxed. These residues are indicated on the 3-D structure in Figure 3a and b. Relative cytotoxic activity (wild type = 100%) was determined using the standard mouse L929 or WEHI assay (Ruff and Gifford, 1981; Espevik and Nissen-Meyer, 1986 respectively), except where indicated otherwise: (a) measured on the TNF-resistant human T24 cell line. (b) measured on the human WiDr cell line. However, no control experiments with TNF were performed on the latter cells so that the relatively high activity of the lymphotoxin mutants on WiDr cells can only be compared with wild type lymphotoxin and not with wild type TNF which tended to score higher in other systems in the same study (Wakabayashi et al., 1990).

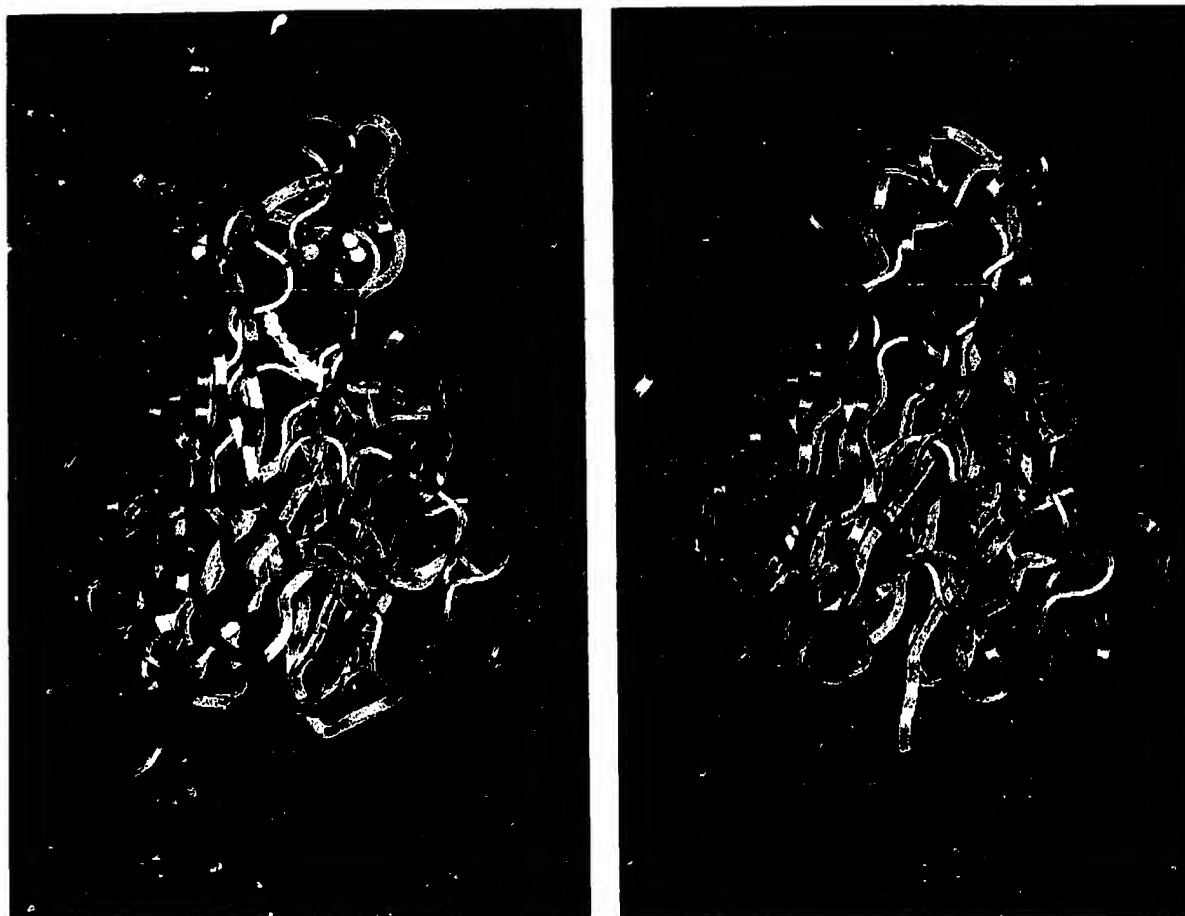


Fig. 2. Ribbon tracing of (a) the human TNF and (b) lymphotoxin trimers. Subunits are coloured red, yellow and blue. The disulphide bonds and cysteine residues in each monomer are visualized.

cytotoxicity assay (Creasey *et al.*, 1987; Geigert *et al.*, 1987; Tsujimoto *et al.*, 1987; Kamijo *et al.*, 1989; Sidhu and Bollon, 1989; Nakamura *et al.*, 1990; our unpublished results). After removal of nine residues, activity drops back to wild type levels (Mark *et al.*, 1987; Yamagishi *et al.*, 1990) and only after deletion of more than ten amino acids, is a decrease in activity observed (Carlino *et al.*, 1987; Creasy *et al.*, 1987; Mark *et al.*, 1987; Nakamura *et al.*, 1991b). A similar feature holds for lymphotoxin where up to 27 N-terminal amino acids could be removed without loss of biological activity (Aggarwal *et al.*, 1985b; Kobayashi *et al.*, 1986; Nishikawa *et al.*, 1990; Wakabayashi *et al.*, 1990). Note that Pro18 of lymphotoxin corresponds structurally to Val1 of TNF (Figure 1).

On the other hand, the C-terminus of TNF seems to be very sensitive, as deletion of eight, three, two and even one amino acids at this end drastically decreases solubility and bioactivity and augments the sensitivity to protease degradation (Sidhu and Bollon, 1989; Gase *et al.*, 1990, 1991). Also in lymphotoxin, the last 16 (Gray *et al.*, 1984) or ten residues (Kobayashi *et al.*, 1986) cannot be removed without drastically reducing the biological activity.

These results can be interpreted on the basis of the 3-D structure, as Lys11 in TNF (Lys28 in lymphotoxin) forms an ion pair with the C-terminal moiety of Leu157 (Leu71 in lymphotoxin) of the adjacent subunit (Sprang and Eck, 1990). Since the receptor binding site of TNF and lymphotoxin is

localized at least in part in the lower half of the molecule (which is the broader half of the pyramidically shaped TNF trimer; see Figure 2a and b), removal of one of the terminal residues or deletion in their close proximity presumably leads to a disturbance of the local (and perhaps even more distal) conformation which could indirectly influence bioactivity. However, in contrast to this hypothesis is the observation of Zhang *et al.* (1992) who noticed that several substitutions at position 11 (K11Q, K11M, K11T, K11N) have no detectable effect on structure and biological activity. It is known that the N-terminus of both cytokines is very flexible and may even curl up and fold into the groove between the subunits (Sprang and Eck, 1992) where it can block the putative active site. Moreover, a murine TNF molecule with an additional ten amino acid stretch at the N-terminus, was devoid of cytotoxic activity (Cseh and Beutler, 1989). The requirement for rigidity of the active site environment is further supported by the observation that substitution of Leu157 by Phe, Met or Gln increases bioactivity (Kamijo *et al.*, 1989; Yamagishi *et al.*, 1990), probably by increasing tighter hydrophobic intersubunit interactions.

A TNF mutant shortened by 17 N-terminal amino acids has proven to be inactive (Nakamura *et al.*, 1990). It is therefore a remarkable claim that addition of the basic amino acid stretch Arg-Ile-Arg-Met at this  $\Delta 17$  mutant would confer on the molecule an even broader cytotoxicity *in vitro* as well as *in vivo* (Soma *et al.*, 1987). Furthermore, the replacement of Asp10 by

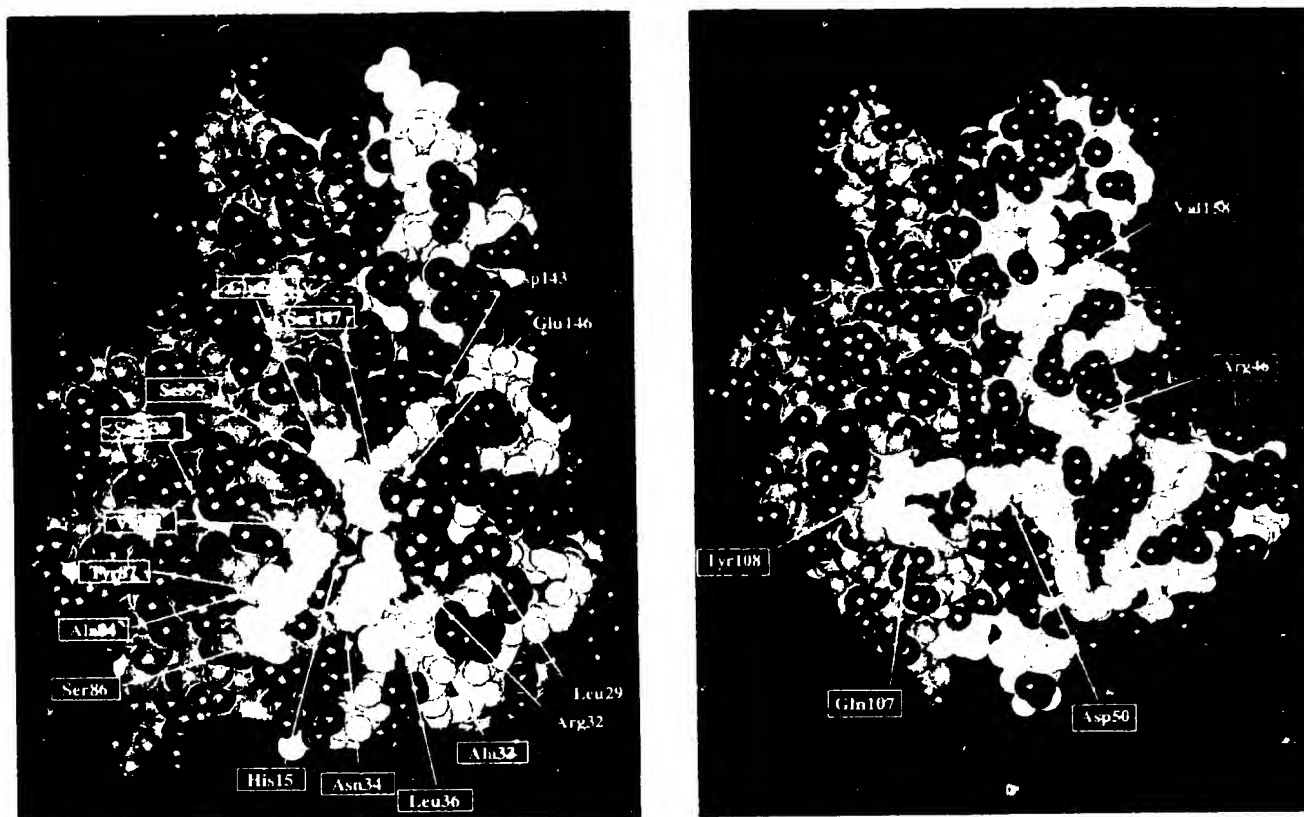


Fig. 3. Space-filling model of (a) human TNF and (b) lymphotoxin. Different subunits are represented in red, yellow and blue. Side chain atoms of the two subunits in front are of darker colour. Relevant amino acids (C $\alpha$  atoms and side chains) which are presumed to be involved in receptor interaction (see Table II) are labelled and marked with white. Side chains that preferentially interact with the TNF-R75 or TNF-R55 are coloured green and magenta respectively.

arginine (=D10R mutation) also caused a broader cytotoxic character, together with the maintenance of solubility and a slightly increased cytotoxicity towards L929 cells (Soma *et al.*, 1988). However, since the sp. act. of the control wild type TNF on these cells was approximately ten times lower than generally accepted ( $1.7 \times 10^6$  versus  $1-5 \times 10^7$  U/mg), the extent of the increase in activity of the mutants towards sensitive and more resistant cells should be re-evaluated and compared to international standardized wild type TNF. Another group reported the inability of the D10R mutant to kill wild type TNF-resistant cell lines, one of which (the bladder carcinoma T24) was the same as in the previous study (Ito *et al.*, 1991) and similar site-specific mutations in the N-terminal region (R2K-S4H-T7H, Kamiyo *et al.*, 1989; P8R-S9K-D10R, Nakamura *et al.*, 1991a) only caused a very small increase in L929 cytotoxicity. It may be noted that cell lines used in different laboratories under the same name, may vary considerably in sensitivity and the use of appropriate standard TNF preparations is highly recommended.

#### Mutagenic analysis

Because originally very few indications were available concerning the location of the active site on the TNF molecule, random mutagenesis on the gene was performed and inactive molecules were selected on the basis of their cytotoxic activity against mouse L-M cells (Yamagishi *et al.*, 1990) or mouse L929 cells (Van Ostade *et al.*, 1991). Starting from these results, additional site-specific mutations were then introduced. The conformation of the resulting TNF analogues was investigated by analysis of solubility and immunoreactivity (Yamagishi *et al.*, 1990) or their solubility, circular dichroism spectra and trimerization properties

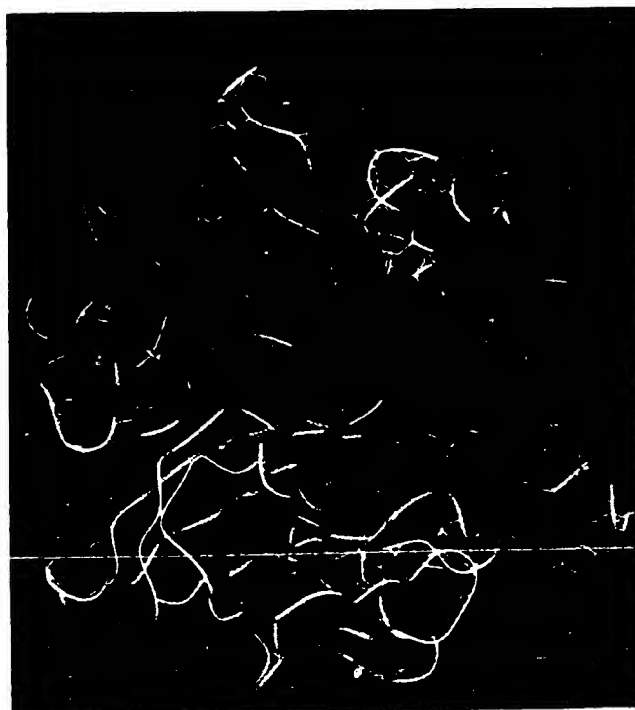


Fig. 4. Ribbon tracing of a bottom-top view of the TNF structure. Different subunits are coloured red, yellow and blue and amino acids putatively involved in receptor interaction are marked white. To make the triplicate active site clearly visible, part of the bottom region was cut off.



(Van Ostade *et al.*, 1991). Results from these two independent studies were quite similar: TNF activity dropped considerably when alterations were introduced in regions 29–36, 84–91, 117–119 and 143–148, without marked change in immunoreactivity or physicochemical characteristics. More specifically, substitutions on the following positions reduced TNF cytotoxicity by a factor of  $>100$ : 29, 32, 33, 34, 36, 84, 86, 87, 91, 117, 119, 143, 146, 147 and 148 (Table II). Except for region 117–119, which is involved in the formation of an inner  $\beta$ -sheet, the other three stretches are all located in loop structures which cluster in the 3-D model of TNF around the cleft, located each time between two subunits of the trimer (Figure 3a and b). The majority of the aforementioned residues are positioned in the lower half of the molecule, near the base of the pyramidal structure, at the outer surface. By convention, the three-sided pyramid is represented with its tip upwards, but when bound to its receptor, the tip is oriented towards the cell surface (Banner *et al.*, 1992).

It is not easy to evaluate to what extent an amino acid substitution in these regions alters the biological activity in either a direct or an indirect manner. Indirect inactivation by destabilizing subunit contacts and, thus, by altering the conformation of the active site cleft, is quite conceivable (Sprang and Eck, 1992). However, Tyr87, for example, is a protruding residue, not involved in intersubunit contacts (Figure 4) and substitution by histidine, asparagine or serine reduces the cytotoxicity drastically (Yamagishi *et al.*, 1990; Zhang *et al.*, 1992). Moreover, the same residue is also involved in the active centre of lymphotoxin where even a conservative change to phenylalanine results in a more than 1000-fold drop in activity (Goh *et al.*, 1992). Hence, Tyr87 could form a direct contact with the TNF receptor, although it has been reported that iodination of TNF and lymphotoxin on this Tyr residue did not reduce the activity (Aggarwal *et al.*, 1985a). Another potential candidate for direct interaction with the receptor is Asp50 in lymphotoxin (position 33 in TNF) because of the large decrease in bioactivity after substitution by conservative amino acids such as asparagine or glutamic acid (Goh *et al.*, 1992). The side chain of this residue clearly points to the exterior (Figure 3b) and, hence, is able to form a direct contact with the receptor. However, the direct involvement in the active centre of TNF of the loop containing Asp50 is still unresolved. Zhang *et al.* (1992) constructed several mutations in this region (L29M, L29V, L29Q, L29P, R31P, R31H, A35P and A35T). They concluded that only the rotation-restricted proline substitutions were able to reduce considerably receptor binding and cytotoxic activity, probably as a result of a local modified conformation of the polypeptide backbone. These results suggest that direct receptor binding sites are more distantly located from the loop connecting  $\beta$ -strands a and a'.

Another random mutagenesis study revealed two potentially relevant TNF mutants: H15Y and P117S (Ito *et al.*, 1991). Although no analysis was made regarding the possible change in conformation of these mutants, the two positions agree well with other reports (position 15, Yamamoto *et al.*, 1987, 1989; position 117, Yamagishi *et al.*, 1990).

It may be noted that although the concentration of the inactivating mutations in a single area constitutes an intrinsic control for the random experiments, the possibility is not excluded that other domains of the molecule are also involved in receptor contact but were not revealed due to conformational disturbances which some substitutions might cause. Thus, while the lower half of the TNF molecule is certainly involved in receptor interaction,

these data do not exclude the possibility that additional sites also play a role. Furthermore, caution must be taken since activity determinations were performed on mouse cell lines (L929 and WEHI) while a species-specific effect can clearly be observed with the majority of the mutants when tested on human cell lines (e.g. Hep-2 and KYM) (Van Ostade *et al.*, submitted). This indicates that the heterologous ligand–receptor interaction is more restricted and, hence, more sensitive to mutagenesis, as compared to the homologous interaction.

Consistent with the aforementioned results are the data of Masegi *et al.* (1990) who showed that mutagenesis in two of the previously mentioned loops (R32E and A84H) could reduce the cytotoxic activity by a factor of  $10^4$ . Zhang *et al.* (1992) also obtained evidence for the possible involvement of Ser95, Ser133 and Ser147, the first of which also lines the intersubunit cleft. Ser133 is located outside the groove and this may suggest an extended receptor binding surface, at least in the lower half of the molecule. However, the activity data of Zhang *et al.* (1992) are in contrast to those reported by Ito *et al.* (1991) and Yamagishi *et al.* (1990) on S95F+G153V and S133I mutants respectively (Table II). Also conflicting results concerning the involvement of the R31H and N39D mutants exist in the reports of Zhang *et al.* (1992) and Yamagishi *et al.* (1990) (Table II). The explanation for these contradictions is still unclear but measurement based on purified proteins versus bacterial lysates could be a possible reason.

Other studies by site-specific mutagenesis were directed at some conserved residues in the primary structures of the two cytokines. For instance, the three histidine residues (positions 15, 73 and 78) of which the first and the last are conserved in TNF and lymphotoxin, were changed to various other amino acids (Table II). Only mutagenesis of His15 caused a clear decrease in bioactivity, but all the mutants (H15N, H15Q, H15K, H15G) accumulated in bacterial inclusion bodies and had to be solubilized by a denaturation–renaturation step (Yamamoto, 1987, 1989). As a consequence, the possibility that inactivity is a result of incorrect refolding of the His15 mutants cannot be excluded. Moreover, Zhang *et al.* (1992) suggested that the putative hydrogen bond between His15 and Tyr59 or the local polar environment in this area is necessary for a correct folding of the monomer. The two tryptophan residues (positions 28 and 114) are not only conserved in all TNF species but also in lymphotoxin (Figure 1). It turned out, however, that replacement by phenylalanine had no pronounced effect on cytotoxic activity (Van Ostade *et al.*, 1988). Also substitution of Trp28 by cysteine did not alter bioactivity, although the mutants W28R, W28L and W28S resulted in abnormal aggregated forms, suggesting a structural role for Trp28 (Zhang *et al.*, 1992). The two cysteines (69 and 101) are conserved in all TNF species and even in bovine and rabbit lymphotoxin but not in human lymphotoxin (Figure 1). As mentioned before, these residues form an intramolecular disulphide bridge, thereby connecting two loops on top of each subunit. This bond was broken by replacing one (Tsujiimoto *et al.*, 1987; Yamagishi *et al.*, 1990; Lin, 1992; unpublished results) or the two Cys residues (Mark *et al.*, 1987; Narachi *et al.*, 1987; Arakawa *et al.*, 1990; Prestrelski and Arakawa, 1991; Lin, 1992). Only a slight reduction in activity was observed in each case and it was reported that the mutant adapted a looser, more flexible structure in solution, as compared to native TNF (Arakawa *et al.*, 1990; Prestrelski and Arakawa, 1991). Moreover, it was observed that crystals, grown either from reduced TNF (Eck *et al.*, 1988) or from the disulphide bridge-deficient mutant C69S (unpublished results) diffract to a high

resolution, but unfortunately were twinned and, thus, unsuitable for X-ray diffraction analysis. Also deletion of a neighbouring amino acid (Gln 102) had no influence on cytotoxic activity (Ito *et al.*, 1991).

#### Other evidence regarding the structure-function relationship

Some theoretical considerations were suggestive for an active centre in the lower half of the molecule. Jones *et al.* (1990b) were able to project onto the TNF structure receptor-interacting residues of viral coat proteins, having a structural similarity to TNF (e.g. picornavirus HRV14 and the foot-and-mouth disease virus FMDV). In the case of Ser133 and the tripeptide Lys128-Gly129-Asp130, even some sequence homology with the corresponding viral residues was noted and, in addition, Ile83 was also a serious candidate. Mutations in the much more related CD40 ligand (see above) that cause non-functional molecules, leading to the disorder of X-linked immunodeficiency with hyper IgM, map onto positions 13 (DiSanto *et al.*, 1993) 18, 19, 130 (Aruffo *et al.*, 1993), 28 (Korthäuer *et al.*, 1993) 43 and 122 (Allen *et al.*, 1993) when projected onto the TNF structure. All these mutations are drastic, non-conservative replacements that are located in the lower half of the molecule and probably disrupt the conformation of the CD40 binding site.

Another approximate localization of the active site came from the determination of the epitopes of neutralizing antibodies. Antibodies were raised against several synthetic TNF fragments and were able to react with the native molecule. However, only antibodies against the N-terminal sequences 1-15 and 1-31 had neutralizing potency while antibodies to epitopes in the middle/top region did not (Socher *et al.*, 1987; Corti *et al.*, 1992b). In a similar study, the last 31 residues of human TNF were replaced by their corresponding mouse amino acids. Such a TNF chimera completely lost a binding site for a neutralizing antihuman monoclonal antibody. Substitution of the only three conserved residues in this stretch, pointed to Arg131 as being involved in the epitope but not in the active centre. As the antibody was neutralizing, residues essential for bioactivity must be located in close proximity to this Arg131 (Tavernier *et al.*, 1990).

Finally, another strategy for active site determination involved the introduction of consensus recognition sequences for N-linked glycosylation in the loop regions of the protein. Expression in Chinese hamster ovary cells (CHO cells) resulted in rationally designed, glycosylated human TNF molecules. These studies learned that introduction of carbohydrate groups on loop regions at the bottom (loops 6-11, 30-34, 38-42, 52-57, 85-89, 125-129 and 155-158), blocked the active centre of TNF. Linkage of glycosyl groups to loops, positioned higher in the molecule had no effect (Leung and Leung, 1992).

#### Mutant TNF molecules that differentially bind to the two TNF receptors

Two TNF receptors (TNF-R55 and TNF-R75) from human (Loetscher *et al.*, 1990; Schall *et al.*, 1990; Smith *et al.*, 1990) and mouse (Barrett *et al.*, 1991; Goodwin *et al.*, 1991; Lewis *et al.*, 1991) have now been cloned. The mouse TNF-R75 does not bind human TNF (Lewis *et al.*, 1991; Tartaglia *et al.*, 1991) and this explains the decreased activity of human TNF towards murine thymocytes or T-cell lines (Plaetinck *et al.*, 1987; Ranges *et al.*, 1988, 1989) which express this receptor type as the major form.

Early mutagenesis studies already suggested a differential activity of some TNF muteins, maintaining the wild type activity in one assay system but showing a reduced activity in another.

The double cysteine mutant, C69A-C101A, had an antiviral activity on HeLa cells comparable to the native protein, but cytolytic activity and inhibition of intracellular lipid production was reduced by a factor of 5-7 (Narachi *et al.*, 1987). Also another double mutant, R31N-R32T, exhibited wild type growth enhancing activity on diploid fibroblast cells, whereas cytotoxic activity towards a myorhabdosarcoma cell line was reduced  $\pm 60$ -fold (Tsujimoto *et al.*, 1987). On the other hand, changing Leu157 (the last residue in TNF and lymphotoxin) to phenylalanine, resulted in a mutant that was five and 20 times more potent in the induction of macrophage differentiation and cytotoxicity respectively, compared to wild type TNF (Kamijo *et al.*, 1989). Although such results have to be evaluated with caution considering the inaccuracy of many cell biological assays, they can be interpreted as cell-specific responses to a signal mediated by one TNF receptor or as differential binding of TNF towards the two TNF receptors TNF-R55 and TNF-R75. The latter possibility was strengthened by the observation that a neutralizing anti-TNF antibody inhibited cytotoxicity and receptor interaction in one tumour model but not in another (Rathjen *et al.*, 1991). We have characterized in detail two TNF mutants, L29S and R32W, which have maintained almost completely the ability to bind to TNF-R55, while the TNF-R75 affinity was decreased drastically (Van Ostade *et al.*, 1993). In addition, this difference in physical binding behaviour towards the two TNF receptors was supported by the biological activities they mediated. In a subsequent study, two mutations at position 29 (L29G and L29Y) were shown to be even more discriminative, as compared to L29S. Also charge reversal at position 146 (E146K) results in a marked differential binding and activity. The results obtained thus far indicate that the TNF-R75-specific region is located in a cluster of three residues at the right side of the groove (Figure 3; Van Ostade *et al.*, submitted). Such an uncoupling of TNF-R55- and TNF-R75-mediated activities could have an interesting potential regarding the clinical use of TNF as an anticancer agent. Indeed, *in vivo* toxicity of human TNF in normal mice is  $\sim 50$  times lower, compared to mouse TNF, while selective tumour toxicity remained unaffected (Brouckaert *et al.*, 1992), suggesting a special role for TNF-R75 in TNF-induced systemic toxicity. Since in the human system the TNF-R55-specific mutants L29S, R32W and E146K could act as the equivalent of human TNF in mice, they offer the prospect of reduced general toxicity, when used in cancer patients. In addition, the R32W and E146K mutants show a decrease in activity towards neutrophils and endothelial cells (Barbara *et al.*, in preparation), two cell types which are believed to play an important part in TNF-induced systemic toxicity (Beutler and Cerami, 1988). Uncoupling of TNF-R55- and TNF-R75-mediated activities was also possible in the mouse system (mouse R37Y; Van Ostade *et al.*, submitted) and, therefore, offers the possibility of testing the efficacy of differentially binding muteins *in vivo* in mice (unlike human TNF, these mouse TNF mutants are expected to be almost non-immunogenic). Furthermore, a reversed differential binding (affinity for TNF-R75 less affected as compared to TNF-R55) and concomitant receptor-mediated activity was observed with several substitutions at position 143 in human TNF (Van Ostade *et al.*, submitted). It thus appears now that both TNF receptors can interact, in addition to a common region, with at least one specific area on the ligand (Figure 3).

#### Difference between TNF and lymph toxin

Certain cell types respond in a different way to TNF and to lymphotoxin (Aggarwal, 1991b and references therein; Porter.

1990 and references therein; Wakabayashi *et al.*, 1990). Both cytokines bind to the purified receptors with similar affinities (Schoenfeld *et al.*, 1991) although on some cell lines a slightly higher affinity for TNF was observed (Aiyer and Aggarwal, 1990; Andrews *et al.*, 1990; Desch *et al.*, 1990; Espevik *et al.*, 1990; Wakabayashi *et al.*, 1990; Raitano *et al.*, 1991). Also remarkable is the weaker binding of lymphotoxin towards the soluble receptor forms, particularly towards TNF-R55 (Sackinger *et al.*, 1989; Engelmann *et al.*, 1990b; Gatanaga *et al.*, 1990; Kohno *et al.*, 1990; Loetscher *et al.*, 1991; Pennica *et al.*, 1992). Obviously, it means that the interaction between the (soluble) receptor and the ligand cannot be identical in molecular detail for TNF and for lymphotoxin. In this respect, it is noteworthy that after alignment with TNF, a deletion and an insertion in lymphotoxin fall within two loops at the top of the trimer (Figure 1; Eck *et al.*, 1992). These loops give the lymphotoxin structure a more flattened (due to the absence of the 103–108 TNF stretch, which is the highest protruding loop in the TNF molecule) and a fan-shaped outlook (insertion of four amino acids between loop residues 70 and 71 in TNF) (Figure 2a and b). Shortening of the latter loop in lymphotoxin (deletion of residues 84–89, lymphotoxin numbering) and, thus, making the molecule more 'TNF-like', resulted in a mutant that apparently showed no drastic change in the other parts of the structure, as determined by circular dichroism spectra and refolding experiments (Wakabayashi *et al.*, 1990). However, this deletion mutant had a remarkably increased activity, corresponding to a higher receptor binding affinity and amino acid replacements in this region could only partially mimic the effect of the deletion. On the other hand, in those assays where TNF was used for comparison, none of the lymphotoxin mutants reached the TNF potency (Wakabayashi *et al.*, 1990). More activity and affinity studies on cells that respond differently to TNF and lymphotoxin need to be done before one can conclude that a deletion in this loop indeed makes lymphotoxin more 'TNF-like' not only in structure but also in activity. If confirmed, the receptor interaction site of both molecules may have to be extended upward.

Thus far, evidence for involvement in the active site of lymphotoxin only points towards residues 50 and 108 and, to a lesser extent, to positions 46, 107 and 158 (lymphotoxin numbering), since substitution at these sites caused a reduction in bioactivity, corresponding with decreased receptor binding (Goh and Porter, 1991, 1992). After superposition onto the TNF structure, these sites (29, 33, 86, 87 and 143 in TNF numbering) are indeed located in loop regions 29–36, 82–91 and 143–148, lining the cleft between the two subunits. In contrast with TNF, however, alterations in neighbouring residues (amino acids 47, 48, 49, 51, 105, 106, 109 and 160; lymphotoxin numbering) did not affect bioactivity. Possibly, the active site of lymphotoxin is not as extended as it is in TNF. Alternatively, the active site region in TNF could be more rigid, such that amino acid substitutions could more easily induce a conformational distortion, indirectly leading to loss of activity.

### The ligand–receptor complex

Not much has been published thus far regarding the 3-D structure of the two TNF receptors. Amino acid sequence comparison allowed a relationship between the external parts of both molecules and those of the nerve growth factor receptor (NGF-R) to be established. Each contains three or four subdomains, characterized by a strongly conserved pattern of cysteine residues (Loetscher *et al.*, 1990; Schall *et al.*, 1990; Smith *et al.*, 1990). The fourth domain (most distal from the cell membrane) of TNF-

R55 seems to be important, although not essential for TNF and lymphotoxin binding, but the first domain is highly necessary (Marsters *et al.*, 1992). The fact that the overall charges of the ligand and of either receptor are opposite (negative and positive respectively) could be an indication of important electrostatic interactions. Since the active form of TNF and lymphotoxin is a trimer (Figure 4) (Arakawa and Yphantis, 1987; Smith and Baglioni, 1987; Wingfield *et al.*, 1987; Lewit-Bentley *et al.*, 1988; Schoenfeld *et al.*, 1991), it follows that binding results in receptor clustering (Loetscher *et al.*, 1991; Pennica *et al.*, 1992). For most if not all TNF activities, this process is sufficient for signal triggering, since monoclonal antibodies (especially IgM) against either of the two receptors were able to mimic TNF activities (Engelman *et al.*, 1990a; Espevik *et al.*, 1990; Shalaby *et al.*, 1990; Tartaglia *et al.*, 1991; Tartaglia and Goeddel, 1992; Vandenabeele *et al.*, 1992). The complex, formed by the attachment of three TNF-R55 receptors to one lymphotoxin trimer, has a rod-like structure (Loetscher *et al.*, 1991) and preliminary X-ray diffraction data indicate that the four receptor subdomains are aligned and interact with discrete regions on the ligand, which points with its top towards the cell membrane (Banner *et al.*, 1992).

### Has TNF other active centres?

#### Interaction with membranes

Thus far, it has been accepted that the active site of TNF was identical to the receptor binding site. Some claims, however, suggesting an additional function of the cytokine have been made. Dependent on lipid composition, TNF concentration and pH, association of TNF with or integration in artificial membranes was demonstrated (Baldwin *et al.*, 1988; Debs *et al.*, 1989; Roozmond *et al.*, 1989). Moreover, high TNF concentrations at low pH values induce an increase in permeability of phospholipid vesicles (Oku *et al.*, 1987), a process that can occur synergistically with interferon- $\gamma$  (Yoshimura and Sone, 1987) and could be the result of integration of TNF into the membrane. In addition, Kagan *et al.* (1992) showed that under approximately the same conditions, the conductance of planar phospholipid membranes increased, which was explained by assuming that Na<sup>+</sup> ions could pass the membrane by flowing through the central channel of inserted intact TNF trimer molecules. How this lipid penetration process takes place is still an open question, but the maintenance of bioactivity, together with decreasing immunoreactivity (Debs *et al.*, 1989), increasing hydrophobicity (possibly by the surface exposure of the two tryptophan residues) and increasing sensitivity to endopeptidase C (Kagan *et al.*, 1992) indicates that conformational rearrangements could occur. Lysosomal membranes are possible targets for this process since receptor-bound TNF becomes internalized and accumulates in the acidic environment of the lysosomes. It has been shown by Ohsawa and Natori (1988) that, after TNF treatment, only TNF-sensitive cells release a permeability-inducing activity for liposomes in the medium. The authors proposed that a specific, TNF-degrading protease was present in these cells and that a resulting degradation product was detected in the medium. Finally, the peripheral localization and partial periplasmatic accumulation of TNF, after its expression in *E. coli* cells, has also been suggested as an argument for an interaction of TNF with membranes (Gase *et al.*, 1991).

One must, however, keep in mind that all well-characterized TNF mutants with reduced biological activity (cytotoxic as well as mitogenic and gene induction activities) show also a drop in

receptor affinity. Furthermore, since thus far there is no report in which a TNF biological effect could not be mimicked by agonistic monoclonal antireceptor antibodies, it may be concluded that, for all the major functions of TNF, receptor clustering explains fully the mechanism of action. However, the possibility that TNF membrane insertion could mediate an as yet undiscovered, biological function is not excluded.

#### Lectin-like binding

In addition to the properties mentioned above, TNF under acidic conditions also shows an affinity for high mannose-substituted glycoproteins (Moonen *et al.*, 1988; Muchmore *et al.*, 1990). It is not known whether this binding is biologically relevant.

#### Activity of peptide fragments

In analogy with the immunostimulatory activity of synthetic peptides from interleukin-1 (Antoni *et al.*, 1986; Mosley *et al.*, 1987), a peptide stretch (positions 31–68) of TNF showed some weak chemotactic stimulatory activity towards fibroblast cells (Postlethwaite and Syer, 1990), but was  $10^4$ -fold less potent than wild type TNF. Despite this very low activity, it remains intriguing that the first part of this peptide (amino acids 31–53) shows a clear monocyte-stimulating activity (Steimer *et al.*, 1992), while the second part (positions 54–68) has some neutrophil-activating capacity (Rathjen *et al.*, 1992). In addition, a cyclic hexapeptide, corresponding to amino acids 127–132 of murine TNF, was reported to cause a very weak cytotoxicity towards several tumour cell lines (Sheh *et al.*, 1990). Obviously, in these cases the signal could not be transmitted through receptor clustering. Whether or not these observations are relevant to an understanding of the true *in vivo* mechanism of action of TNF remains to be proven.

#### TNF hybrids

The genetic coupling of lymphotoxin (mutant  $\Delta 1-23$ ) or TNF (mutant S5T + D10R) mutants to interferon- $\gamma$  (Feng *et al.*, 1988) and thymosin- $\beta 4$  (Tsuji *et al.*, 1989) respectively, resulted in chimeras with an increased or unchanged cytotoxic activity *in vitro* respectively. The augmentation of cytotoxicity of the former construct possibly resulted from the synergistic effect, well known for these cytokines (Sugarman *et al.*, 1985; Fransen *et al.*, 1986). The usefulness of the TNF/thymosin- $\beta 4$  hybrid was further investigated in tumour-bearing mice (Noguchi *et al.*, 1991). Also a chimeric human/mouse antitransferrin receptor antibody-Fab fragment was linked to human TNF and showed cytotoxic activity as well as transferrin receptor binding characteristics on tumour cell lines (Hoogenboom *et al.*, 1991a, b). Although these constructs had apparently retained immunoreactivity with at least some anti-TNF monoclonal antibodies, the actual oligomeric state of the TNF part remains to be elucidated and caution must be used in the interpretation of the activity data. More particularly, one wonders what kind of structure results from coupling the TNF polypeptide, which normally trimerizes, to an interferon- $\gamma$  or an immunoglobulin heavy chain polypeptide, which normally dimerizes. Also immunogenicity of such chimeras would be a cause of concern.

#### Conclusion

At least part of the receptor binding site of TNF is located in the lower half of the conically shaped trimeric structure. This can be concluded not only from mutagenesis analysis, but also from deletion studies at the N- and C-terminal ends, blocking experiments with antibodies or carbohydrate groups and from comparison with the receptor binding sites of related viral coat

proteins. Random and site-specific mutagenesis showed that amino acids involved in receptor binding of TNF, are located at each interface between two subunits, particularly loops 29–36, 84–91 and 143–148. Consistent with these observations are the positions of the active site residues of lymphotoxin, since they correspond with the three loops in TNF after superposition of the two structures. The groove between the subunits is repeated three times in the TNF and lymphotoxin trimer. Hence, one TNF or lymphotoxin molecule has three receptor binding sites and acts by clustering three receptors, an event that is sufficient, at least for the large majority of TNF effects, to transmit the TNF signal. Also, TNF mutants that bind selectively to one of the two receptors have been developed. Considering that the intracellular domains of the two TNF receptors are totally unrelated and that the two receptors have a different cell distribution and regulation, these receptor-restricted TNF mutants offer the potential to uncouple several TNF-mediated effects. More specifically, based on *in vivo* results in the murine system, one may hope that TNF mutants which only react with TNF-R55 would have retained the beneficial antitumour effects, but be at least partially devoid of *in vivo* deleterious effects. Detailed information at the atomic level regarding the interaction of TNF with either of its two receptors has yet to come from crystal structures of ligand-receptor complexes. This, together with further protein engineering, should permit an understanding of the fine details of TNF and lymphotoxin and their interactions with their two receptors.

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# STIC-ILL

**From:** Romeo, David  
**Sent:** Monday, August 30, 1999 2:35 PM  
**To:** STIC-ILL  
**Subject:** 08/981,559 Pennica D\*\*\* ; Nedwin G E; Hayflick J S; Seeburg P H; Derynck R;

Borrower's Name ... David Romeo  
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Palladino M A; Kohr W J; Aggarwal B B; Goeddel D V  
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factor (TNF)<sup>32,35-41</sup>. This factor causes necrosis of MethA(a) sarcoma *in vivo*; it is also active in causing lysis of murine L-929 cells *in vitro*<sup>32,39</sup>. TNF activity on L-929 cells is not inhibited by neutralizing antibodies specific for lymphotoxin<sup>13</sup>, however, and appears to have distinct biochemical properties<sup>39,40</sup>. Furthermore, the lymphotoxin cDNA probe failed to hybridize on Northern blots to mRNA from induced macrophages producing cytolytic activity (Fig. 2). The isolation of a TNF cDNA clone further demonstrates that lymphotoxin and TNF are distinct molecules (see accompanying article<sup>42</sup>).

A cytolytic factor derived from B-cell lines which displays *in vitro* and *in vivo* anticellular activity has been described<sup>43</sup>; this has been designated 'tumour necrosis factor' based on its activity in the MethA sarcoma assay. This activity probably results from lymphotoxin, however, because it has similar biological activities, biochemical properties and is made by the cell line (RPMI-1788) used in this study for the purification of lymphotoxin. Natural killer cells also can be induced to secrete an anticellular factor<sup>44,45</sup>. The lymphotoxin gene probe and lymphotoxin-specific antibodies will be useful in determining the

relationship of this natural killer cell cytotoxic factor to lymphotoxin.

Lymphotoxin has been reported to act synergistically with  $\alpha$ -interferon<sup>46</sup> and  $\gamma$ -interferon<sup>13,16,47</sup> *in vitro* and *in vivo*. The potent antitumour activity of  $\gamma$ -interferon or lymphotoxin in natural preparations may be a result of the synergistic activity when both lymphokines are present<sup>13</sup>. The ready availability of lymphotoxin produced via recombinant methods will aid the biological characterization of this anticellular lymphokine. It will also help to define the antitumour mechanism of lymphotoxin, as well as its role *in vivo* in the regulation of the immune system and its interaction with other lymphokines.

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## Human tumour necrosis factor: precursor structure, expression and homology to lymphotoxin

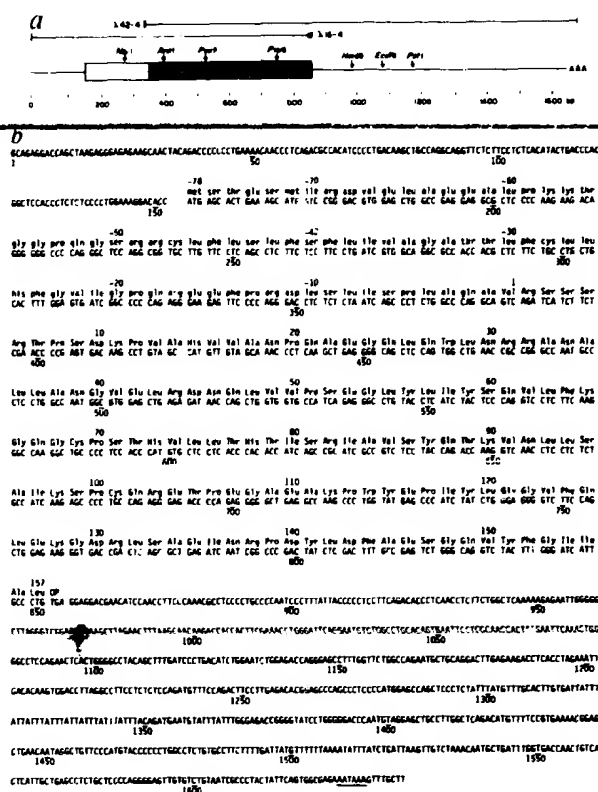
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*Human tumour necrosis factor has about 30% homology in its amino acid sequence with lymphotoxin, a lymphokine that has similar biological properties. Recombinant tumour necrosis factor can be obtained by expression of its complementary DNA in Escherichia coli and induces the haemorrhagic necrosis of transplanted methylcholanthrene-induced sarcomas in syngeneic mice.*

TUMOUR necrosis factor (TNF) has been associated with *in vitro* and *in vivo* killing of tumour cells. This activity was discovered originally in the sera of mice and rabbits injected first with *Mycobacterium bovis* strain bacillus Calmette-Guérin (BCG) or other immunostimulatory agents, and subsequently

with endotoxin<sup>1,2</sup>. Serum from such animals causes haemorrhagic necrosis and in some cases complete regression of certain transplanted tumours in mice<sup>1,2</sup>. TNF-like activity has also been detected in the media of BCG/endotoxin-induced monocyte cultures (reviewed in ref. 2) and mitogen-stimulated peripheral



**Fig. 1** TNF cDNA sequences and predicted amino acid sequence. **a**, Schematic representation of human TNF cDNA clones. Overlapping clones 142-4 and 116-4 used in sequence determination and a schematic diagram of the complete cDNA structure are shown. Line, untranslated sequences; boxes, coding sequences; white portion, sequences encoding the signal peptide; shaded regions code for mature TNF. The black box on the 3' end of clone 116-4 indicates that this clone was obtained by specific priming. **b**, Nucleotide sequence and deduced amino acid sequence of human TNF cDNA. Numbers above each line refer to amino acid positions and numbers below each line refer to nucleotide positions. The amino acid labelled 1 represents the first amino acid of mature TNF<sup>16</sup>. The 76 amino acids preceding this position are indicated by lower case lettering. Sequence underlined indicates the polyadenylation recognition site<sup>27</sup>.

**Methods:** **a**, Total RNA was extracted<sup>20</sup> from HL-60 cultures 4 h after PMA induction and poly(A)-containing RNA was purified on oligo(dT)-cellulose<sup>43</sup>. Double-stranded cDNA was prepared by oligo(dT) priming<sup>19</sup> using 7.5 µg mRNA and fractionated on a 6% polyacrylamide gel. 700 ng cDNA >600 bp was recovered by electroelution. Synthetic *Eco*RI adaptors<sup>44</sup> were ligated to 20 ng cDNA before ligating into  $\lambda$ gt10 (ref. 26). 200,000 cDNA clones were obtained. The same conditions were used to prepare a specifically-primed cDNA library of 200,000 clones using as primer the hexadecanucleotide dTGGATGTTCTGCTCTCC (complementary to nucleotides 853-870). Plaque screening<sup>45</sup>, <sup>32</sup>P-radiolabelling of synthetic 42-mer probe<sup>25</sup> and hybridizations<sup>22</sup> were performed. **b**, DN<sub>A</sub> sequencing was performed by the dideoxynucleotide chain termination procedure<sup>46-48</sup>. The cDNA insert of 142-4 consists of nucleotides 337-1643 and the cDNA insert of 116-4 consists of nucleotides 1-870.

blood leukocytes (PBLs)<sup>3</sup>.

TNF activity is cytolytic or cytostatic against many transformed cell lines *in vitro* without obvious species specificity, yet has no known effect on normal mouse embryo fibroblasts or non-transformed cell lines<sup>1,2,4-8</sup>. Activated macrophages may constitute the major cellular origin of TNF<sup>1,5,9,10</sup>, providing an important criterion for distinguishing this factor from the lymphoid cell-derived cytotoxin, lymphotoxin<sup>11</sup>. The primary structure of lymphotoxin was determined recently by protein sequencing<sup>12</sup> and complementary DNA cloning (see accompanying article<sup>13</sup>).

**Table 1** Human TNF production by various cell populations and cell lines

Cell source	Inducing agent(s)	Cytotoxic activity (U ml <sup>-1</sup> )	
		TNF	Lymphotoxin
Unfractionated PBLs	None	<8	<8
	LPS	20	<8
	BCG	82	<8
	BCG/LPS	86	<8
	BCG/LPS/PMA	140	<8
	PMA	280	<8
PBLs (adherent cells)	SEB/Ta <sub>1</sub>	100	10
	SEB/Ta <sub>1</sub> /PMA	1,600	200
	None	<8	<8
	BCG/LPS	350	<8
	SEB/Ta <sub>1</sub> /PMA	590	<8
	None	<8	<8
PBLs (non-adherent cells)	BCG/LPS	<8	<8
	SEB/Ta <sub>1</sub> /PMA	<8	350
	None	<8	<8
HL-60	None	<8	<8
	PMA	380	<8
U-937	None	<8	<8
	PMA	32	<8

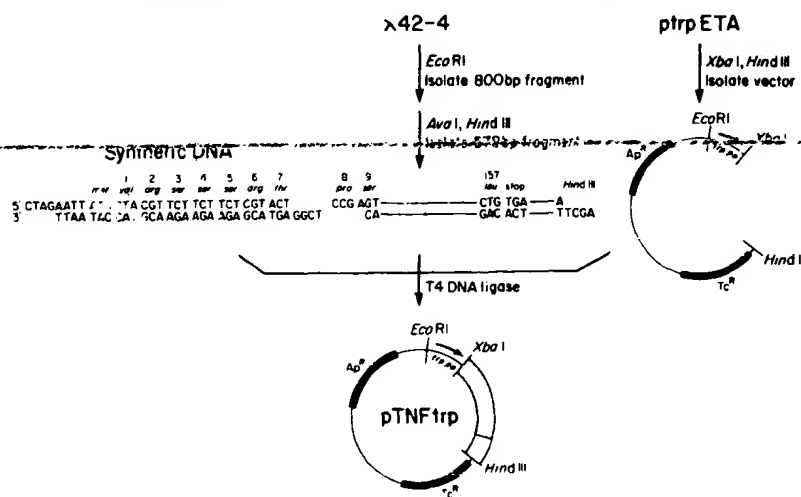
PBLs were obtained from plateletpheresis residues (Boston Red Cross) by Ficoll-Hypaque centrifugation<sup>3</sup>. Separation of PBLs into adherent and non-adherent populations was performed as described previously<sup>11</sup>. HL-60 (CCL 240) and U-937 cell lines (CRL 1593) were obtained from the American Type Culture Collection. Cells were suspended at  $5 \times 10^6$  cells ml<sup>-1</sup> in RPMI 1640 media containing 10% fetal bovine serum. Cultures were induced with one or more of the following agents:  $2 \times 10^5$  organisms per ml of BCG (Calbiochem-Behring),  $20 \mu\text{g ml}^{-1}$  *Salmonella typhimurium* lipopolysaccharide (LPS, Sigma),  $1 \mu\text{g ml}^{-1}$  staphylococcal enterotoxin B (SEB, Sigma),  $1 \mu\text{g ml}^{-1}$  thymosin  $\alpha_1$  (Ta<sub>1</sub>)<sup>42</sup> and  $10 \text{ ng ml}^{-1}$  PMA (P-L Biochemicals). Cell-free supernatants were collected 24 h after induction except for the BCG/LPS and BCG/LPS/PMA treatments; for these two inductions a 24-h BCG stimulation was followed by an additional 24-h treatment with LPS and LPS/PMA, respectively. Samples were assayed for cytotoxic activity on mouse L-929 fibroblasts as described previously<sup>11</sup>. The activities shown represent TNF-specific or lymphotoxin-specific units as determined after antibody neutralization at 4 °C for 4 h before assay. The units indicated were obtained from one representative donor in the case of the PBLs and from a single experiment when cell lines were used. Rabbit anti-human TNF antiserum was prepared against partially purified TNF from PBLs (L. Svedersky and T. Bringman, unpublished results). Rabbit anti-human lymphotoxin antiserum was prepared against purified human lymphotoxin from RPMI 1788 lymphoblastoid cells<sup>11</sup>.

Here we identify a cell line with monocyte-like characteristics providing a source for human TNF and its messenger RNA. cDNA clones were isolated that encode a polypeptide related structurally to lymphotoxin. This cDNA was engineered to direct the synthesis of a relative molecular mass ( $M_r$ ) 17,000 protein in *E. coli* with the immunological characteristics as well as *in vitro* and *in vivo* biological properties of natural human TNF.

### A human TNF-producing cell line

We isolated PBLs by Ficoll-Hypaque density centrifugation and fractionated them into adherent monocytic and non-adherent lymphocytic fractions. After stimulation with BCG and endotoxin (lipopolysaccharide, LPS), we detected an activity cytotoxic to murine L-929 cells in the culture media of unfractionated mononuclear cells and monocytes (Table 1). No cytotoxic activity was produced by the non-adherent cells following the same BCG/LPS induction procedure. The failure of rabbit anti-human lymphotoxin antibodies to neutralize the cytotoxic activity demonstrates its difference from lymphotoxin. Moreover, the results of previous *in vivo* studies using BCG/LPS induction procedures<sup>1,2</sup> demonstrate that the activity can probably be attributed to TNF. Antiserum raised against partially purified PBL-produced TNF completely neutralized this activity (Table 1).

**Fig. 2** Construction of a plasmid coding for the direct expression of mature human TNF in *E. coli*. The recombinant phage  $\lambda$ 42-4 (10  $\mu$ g) was digested with *Eco*RI and the 800-bp fragment containing the entire TNF coding region was isolated. The digestion with *Ava*I and *Hind*III gave a 578-bp fragment coding for amino acids 8-157. Two synthetic complementary deoxyoligonucleotides<sup>17</sup>, 5'-dCTAGAAT-TATGGTACGTTCTTCTCTCGTACT and 5'-dTCGGAGTACGAGAAGAAGAACGTA-CCATAAT, were designed to code for amino acids 1-7 of TNF, preceded by an ATG translational initiation codon, and to contain an *Xba*I cohesive terminus. The choice of codons for the first six amino acids of TNF was based on *E. coli* codon usage preferences<sup>49</sup>. An AATT sequence was incorporated upstream of the ATG to maximize expression by giving optimal spacing between the initiation codon and the *trp* leader Shine-Dalgarno sequence<sup>50</sup>. The pBR322-derived plasmid pTrpETA<sup>51</sup> was cleaved with *Hind*III and *Xba*I and the large fragment recovered by electroelution. The *Ava*I-*Hind*III fragment and the two synthetic deoxyoligonucleotides were inserted into the plasmid pTNFtrp expression vector to give the plasmid pTNFtrp. The methods used to assemble the fragments and verify the construction of pTNFtrp have been described previously<sup>19,20</sup>. *E. coli* W3110/pTNFtrp was grown in M9 medium containing 5  $\mu$ g ml<sup>-1</sup> tetracycline to 0.2  $A_{550}$  units. Indole acrylic acid was added to a concentration of 20  $\mu$ g ml<sup>-1</sup>. The cells were collected at  $A_{550}$  = 1.0 and washed with cold PBS. The final cell pellet was resuspended in 1 ml PBS, sonicated on ice for 30 s and the resulting extract diluted in PBS for assay on L-929 cells<sup>11</sup>.



Yields of adherent cells from peripheral blood were low and the levels of TNF produced were variable and donor-dependent; we therefore tested alternative induction schemes for the production of TNF from total PBLs (Table 1). An increase in cytotoxic activity was observed when the PBLs were co-stimulated with *Staphylococcus* enterotoxin B, desacetyl-thymosin- $\alpha_1$  and the tumour-promoting agent 4 $\beta$ -phorbol 12 $\beta$ -myristate 13 $\alpha$ -acetate (PMA). However, antibody neutralization experiments demonstrated that a significant portion of measured activity was lymphotoxin. Therefore, we screened a number of transformed cell lines of haematopoietic origin for their ability to synthesize TNF. Activity which could be neutralized by anti-TNF antiserum was detected following PMA treatment in two monocytic-like cell lines, HL-60, derived from a promyelocytic leukaemia<sup>14</sup>, and U-937, derived from a histiocytic lymphoma<sup>15</sup> (Table 1). The HL-60 cell line consistently produced higher TNF titres (300-400 U ml<sup>-1</sup> 24 h after induction) than the U-937 cell line (<100 U ml<sup>-1</sup>). A time course of TNF synthesis by HL-60 cultures indicated that measurable activity was detected 2 h after PMA treatment (data not shown). Therefore the HL-60 cell line was selected for future experiments; supernatants were collected 16-24 h after induction for protein purification and 4-h inductions were used when cells were collected for RNA isolation.

### TNF cDNA clone identification

Human TNF was purified to homogeneity from filtrates of PMA-stimulated HL-60 cell cultures (see ref. 16). A single component of  $M_r$  17,000 was observed when the purified TNF was analysed by SDS-gel electrophoresis in reducing conditions. To obtain amino acid sequence information, tryptic peptides of TNF were prepared and separated by reverse-phase HPLC.

The preliminary sequence Glu-Thr-Pro-Glu-Gly-Ala-Glu-Ala-Lys-Pro-Trp-Tyr-Glu-Lys was determined for the first tryptic fragment (TD-6) analysed. A single synthetic 42-base long deoxyoligonucleotide (42-mer) which could code for this amino acid sequence was chemically synthesized<sup>17</sup> for use as a hybridization probe. The design of the probe sequence (dGAAACCCCT-GAAGGGGCTGAAGCCAAGCCCTGGTATGAAAAG) was based on published human codon usage frequencies<sup>4</sup> and the codon bias of human  $\gamma$ -interferon<sup>19</sup>, tissue-type plasminogen activator<sup>20</sup> and lymphotoxin<sup>11</sup>. The general usefulness of this 'long probe' approach has been demonstrated recently by the identification of several cloned genomic DNA sequences<sup>21-23</sup> and cDNAs<sup>24,25</sup>.

An oligo(dT)-primed HL-60 cDNA library of ~200,000 clones prepared in  $\lambda$ gt10 (ref. 26) was screened with the <sup>32</sup>P-labelled 42-mer. The nine distinct phage which gave positive signals with this probe were hybridized with 'induced' and 'non-induced' <sup>32</sup>P-labelled cDNA probes<sup>19</sup> prepared using poly(A) mRNA obtained from 4-h PMA-treated and untreated HL-60 cultures, respectively. Seven of these recombinant phage DNAs hybridized weakly to the induced probe but did not hybridize to the uninduced probe, as expected for authentic TNF cDNAs. Restriction endonuclease mapping indicated that these seven cDNA clones were related to each other and that the phage  $\lambda$ 42-4 contained the longest cDNA insert.

### TNF cDNA sequence

We determined the sequence of the 1,300 base pair (bp) cDNA insert of phage  $\lambda$ 42-4 (nucleotides 337-1,643; Fig. 1). Alignment of the cDNA sequence with the 42-mer probe sequence gave the proper reading frame of the cDNA and demonstrated that it did indeed encode TNF. Of the 14 amino acids (residues 104-117, Fig. 1) assigned to tryptic peptide TD-6 on the basis of preliminary protein sequence, 13 were correct; the only discrepancy was in the last amino acid (position 117) where the cDNA sequence encodes a proline residue rather than the predicted lysine. Despite this difference, the hybridization of the synthetic probe to the TNF cDNA clone was successful, as the 42-mer matched the cDNA sequence in 34 of the first 38 positions, including a stretch of 17 consecutive homologous nucleotides (nucleotides 711-727; Fig. 1).

The assignment of valine (residue 1, Fig. 1) as the first residue of mature TNF was based on NH<sub>2</sub>-terminal protein sequence analysis of the intact molecule (Val-Arg-Ser-Ser-Ser-...)<sup>16</sup>. There are 156 amino acids encoded after this valine before an in-phase termination codon occurs. The coding region of TNF is followed by 792 nucleotides of 3' untranslated sequence containing the hexanucleotide AATAAA (position 1,630-1,635) which precedes the site of polyadenylation in most eukaryotic mRNAs<sup>27</sup>.

Additional confirmation that this sequence codes for TNF was obtained by determining the amino acid sequence of nine tryptic peptides of natural HL-60 TNF and several peptides generated by digestion with *S. aureus* V8 protease and chymotrypsin<sup>16</sup>. The  $M_r$  of 17,356 calculated for the mature TNF monomer from the cDNA sequence agrees closely with the value obtained for natural TNF by SDS-polyacrylamide gel electrophoresis and amino acid composition<sup>16</sup>. These results and

**Table 2** Necrosis of MethA sarcoma *in vivo* by TNF

Treatment	Necrotic response			
	+++	++	+	-
	No. of mice			
PBS, i.l.	0	1	1	9
PBS, i.p.	0	0	1	4
PBS, i.m.	0	0	1	4
<i>E. coli</i> LPS, i.l.	0	0	1	9
HL-60 TNF, i.l.	5	1	1	0
rTNF, i.l.	7	5	0	0
rTNF, i.p.	2	1	2	0
rTNF, i.m.	2	2	1	0

(BALB/c×C57BL/6)F<sub>1</sub> female mice were injected intradermally with  $5 \times 10^5$  BALB/c MethA sarcoma cells. Ten days later, the tumours (0.75 cm average diameter) were injected intraleisionally (i.l.,  $1 \times 10^5$  U), intraperitoneally (i.p.,  $5 \times 10^5$  U) or intramuscularly (i.m.,  $5 \times 10^5$  U) with TNF in a total volume of 0.1 ml PBS. At 24 h after TNF treatment the tumours were excised, sectioned and scored for haemorrhagic necrosis by visual and histological examination as described previously<sup>1</sup>. In the maximum response (+++) 50–75% of the tumour mass is markedly necrotic after 24 h; ++ denotes a moderate response, that is 25–50% haemorrhagic necrosis; +, a minimal response of <25% haemorrhagic necrosis; –, tumours showed no visible necrosis. Natural TNF was purified from HL-60 cultures as described elsewhere<sup>10</sup>. Recombinant TNF (rTNF) was purified from *E. coli* W3110/pTNFtrp to a purity of >95% and a specific activity of  $\sim 10^8$  units  $\text{mg}^{-1}$  (T. Bringman, unpublished results).

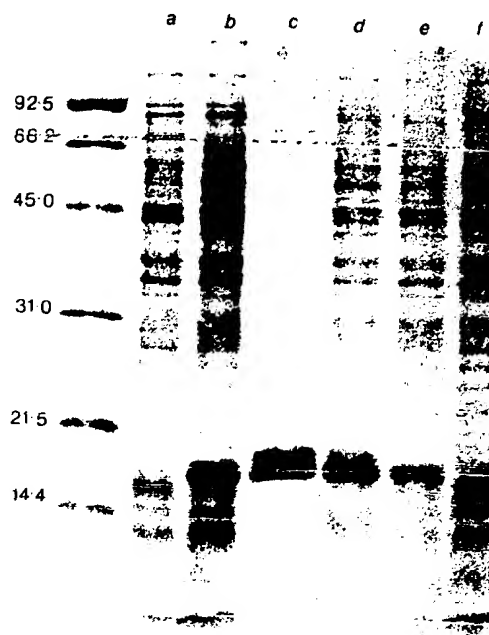
the absence of any potential N-glycosylation sites in the deduced amino acid sequence suggest that TNF is not a glycoprotein. These data suggest also that TNF may occur naturally in multimeric form, as the *M<sub>r</sub>* estimated previously for human TNF ranged from 34,000–140,000 (refs 6, 28). There are two cysteine residues (positions 69 and 101) in TNF which are probably involved in a single intramolecular disulphide bond<sup>10</sup>.

The cDNA clone  $\lambda$ 42-4 contains the entire coding region of mature TNF but lacks a complete signal peptide coding sequence and initiation codon. To obtain the missing sequence information, a specifically-primed cDNA library was prepared (see Fig. 1 legend) and screened with the <sup>32</sup>P-labelled  $\lambda$ 42-4 cDNA insert. A cDNA clone ( $\lambda$ 16-4) was identified which contained an insert extending 337 bp further 5' than the  $\lambda$ 42-4 insert (Fig. 1).

From an analysis of the TNF cDNA sequence, it seems that TNF is synthesized initially as part of a larger precursor (pre-TNF). Starting at the 5' end of the cDNA, 125 nucleotides of non-translated sequence are followed by a methionine codon and an open reading frame of 233 amino acids. This AUG is preceded by termination codons in all three frames, suggesting that it is the initiation codon. Furthermore, the sequence context of this AUG conforms closely to the CC<sup>+</sup>CCAUGG proposed<sup>29</sup> as a consensus sequence for eukaryotic initiator sites.

The presequence of 76 residues is most probably involved in the secretion of TNF as it is not observed on the mature TNF polypeptide and contains an unusually long hydrophobic region of 26 amino acids (residues –46 to –21). Typically, signal peptides involved in protein secretion are only 20–30 amino acids long<sup>30,31</sup>. However, a signal sequence for the Rous sarcoma virus envelope glycoprotein<sup>32</sup> is atypically long (63 residues) and contains also many charged amino acids at its amino terminus, such as pre-TNF. It is interesting to note the presence of Arg-Arg and Lys-Lys dipeptides in the first 30 amino acids of the TNF pre-sequence, as pairs of basic amino acids often serve as cleavage sites for the release of physiologically-important peptides from precursor molecules<sup>33–36</sup>.

We used the <sup>32</sup>P-labelled  $\lambda$ 42-4 cDNA insert to examine TNF gene structure and mRNA size. Results from Southern<sup>37</sup> hybridizations indicate that only a single gene for TNF is present in the human genome. Northern hybridization analysis<sup>38</sup> shows that a single mRNA species  $\sim 1.8$  S in size is synthesized in PMA-induced HL-60 cultures and BCG/LPS-treated macrophages isolated from human PBLs. This provides additional evidence that the same cytotoxin is produced from both



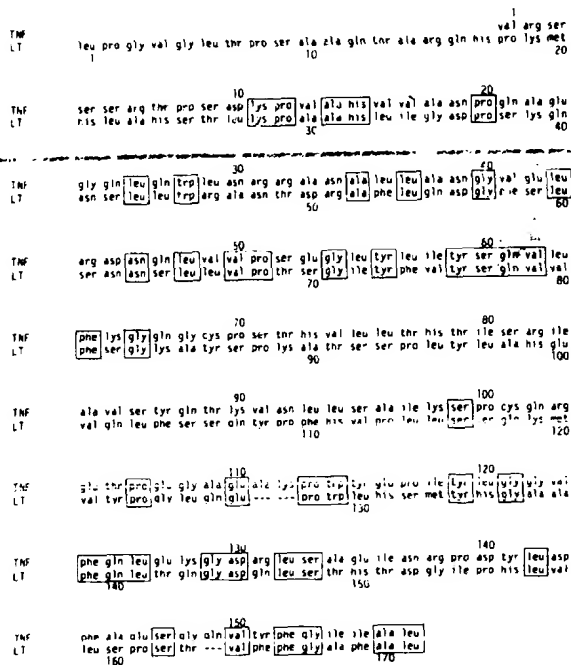
**Fig. 3** SDS-polyacrylamide gel electrophoresis of human TNF synthesized in *E. coli*. *E. coli* K-12 strain W3110, transformed with pTNFtrp or pBR322, was grown in M9 medium containing  $5 \mu\text{g ml}^{-1}$  tetracycline. Cells were collected, lysed in 2% SDS, 1%  $\beta$ -mercaptoethanol and precipitated with 10 volumes of cold acetone. Samples were electrophoresed on a 12.5% SDS-polyacrylamide slab gel using the buffer system of Maizels<sup>32</sup> and the gel stained with Coomassie brilliant blue. The left lane contains protein *M<sub>r</sub>* standards ( $\times 10^{-3}$ ): phosphorylase b (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500) and lysozyme (14,500). Lanes a, f, cell lysates of *E. coli* W3110/pBR322; lanes b, e, cell lysates of *E. coli* W3110/pTNFtrp; lane c, partially purified human TNF isolated from the HL60 cell line<sup>10</sup>; lane d, mixture of the *E. coli* W3110/pTNFtrp cell lysate and the HL60-derived, purified TNF.

cell sources and suggests that the TNF cDNA sequence shown in Fig. 1 represents a nearly full-length copy of TNF mRNA. No hybridization was detected to mRNA isolated from uninduced cultures (data not shown).

### TNF synthesis in *E. coli*

Proof that the cDNA described here encodes TNF requires the demonstration that it can direct the synthesis of a gene product with the properties of authentic human TNF. To allow characterization of the protein encoded by the cloned cDNA, we engineered the TNF cDNA sequence for direct expression in *E. coli* (Fig. 2). In the resulting expression plasmid, pTNFtrp, the TNF DNA sequence is under the transcriptional control of a 300-bp DNA fragment of the *E. coli* *trp* operon containing the *trp* promoter, operator and Shine-Dalgarno sequence of the *trp* leader peptide.

Total extracts of *E. coli* K-12 strain W3110 transformed with pTNFtrp contained a prominent polypeptide with an apparent *M<sub>r</sub>* 17,000 (Fig. 3, lanes b, e). This protein is not visible in cells transformed with pBR322 (lanes a, f), strong evidence that it represents the translational product of the TNF cDNA sequence. Furthermore, this protein co-migrates with authentic TNF (lane c) isolated from the HL-60 cell line (lane d), suggesting that no significant post-translational processing of TNF occurs in the HL-60 cell line. This is unlike lymphotoxin and  $\gamma$ -interferon, both of which occur naturally as heterogeneous glycoproteins as a consequence of N-terminal<sup>12</sup> and C-terminal<sup>19</sup> proteolysis, respectively.



**Fig. 4** Comparison of the amino acid sequence of human TNF with human lymphotoxin<sup>12,13</sup>. The sequences have been aligned to give maximal homology by introducing two gaps (indicated by dashed lines) into the lymphotoxin sequence. Identical amino acids are boxed. The numbers above each row (1-157) and below each row (1-171) indicate the amino acids of mature TNF and lymphotoxin (LT), respectively.

Verification of the bacterial production of biologically-active TNF was obtained by assaying extracts of *E. coli* W3110/pTNFtrp for cytolytic activity in the murine L-929 fibroblast assay<sup>11</sup>. Approximately 300,000 units of activity were detected per ml of culture at  $A_{550} = 1$ , whereas no activity was observed in *E. coli* W3110/pBR322 controls. This corresponds to ~3 mg of active TNF per l ( $A_{550} = 1$ ) or about 300,000 molecules of active TNF per cell if a specific activity of  $10^6$  units  $\text{mg}^{-1}$  (ref. 16) is assumed. The activity was neutralized by antiserum prepared against partially purified PBL-derived TNF, but was not neutralized by preimmune serum or rabbit anti-human lymphotoxin antibodies (data not shown).

### *In vivo* necrosis activity

TNF is generally defined as a cytotoxin released by BCG/LPS-treated macrophages which induces the haemorrhagic necrosis of methylcholanthrene-induced (MethA) sarcomas in BALB/c mice<sup>1,2</sup>. Therefore, we examined recombinant human TNF purified from *E. coli* and natural human TNF from PMA-induced HL-60 cultures for *in vivo* tumour necrosis activity in the MethA assay<sup>1</sup>. Both recombinant and natural TNF samples elicited significant necrotic responses, regardless of whether the TNF was injected intralesionally or systemically (Table 2). Minimal or no necrosis of the MethA sarcoma tumours was observed in mice injected with either phosphate-buffered saline (PBS) or 100  $\mu\text{g}$  *E. coli* LPS. These results, taken with the antibody neutralization and Northern hybridization data, provide further evidence that the cytotoxin described here is human TNF.

### Homology to lymphotoxin

The known *in vivo* and *in vitro* biological activities of TNF and lymphotoxin are very similar<sup>2,3,13</sup>. TNF and lymphotoxin are now known to be antigenically distinct molecules<sup>1</sup>. It has thus become common to distinguish these two lymphokines on the basis of the cell populations responsible for their synthesis. We have compared the amino acid sequences of human TNF and

lymphotoxin to determine whether the similarities in their biological properties might be attributed to common structural features (Fig. 4). By introducing two gaps, the lymphotoxin sequence can be aligned with the TNF sequence so that distinct homologies are apparent; we find 44 of the 157 TNF residues (28%) are identical to corresponding lymphotoxin amino acids with many of the remaining differences between the two polypeptides resulting from conservative amino acid changes. The nucleotide homology over this coding region is 46% (data not shown). Two particularly conserved regions occur at amino acids 35-66 and 110-133 (TNF numbering) where 50% of the residues (28 of 56) are identical for TNF and lymphotoxin. The hydrophobic carboxy-termini of the two molecules are also significantly conserved. It is probable that the conserved regions are crucial to the shared cytotoxic activities of TNF and lymphotoxin, perhaps through interaction with a common receptor expressed on the surface of transformed cells. Support for this hypothesis is provided by the lack of cytotoxic activity in a truncated lymphotoxin polypeptide lacking its last 16 amino acids<sup>13</sup>.

Lymphotoxin has 18 more  $\text{NH}_2$ -terminal amino acids than TNF (Fig. 4), suggesting that this region is not required for cytotoxic activity. In fact, a 148 residue lymphotoxin, consisting of amino acids 24-171 of mature lymphotoxin, and having similar cytotoxic effects on L-929 cells, has been isolated from the RPMI-1788 cell line<sup>11,12</sup>. It is also interesting that amino acids 67-109 of TNF are unrelated to the corresponding region of lymphotoxin; only two of 43 residues are identical. This region includes all of the amino acids spanned by the Cys 69-Cys 101 disulphide bridge of TNF. One possible role for this non-conserved region could be to position correctly the two surrounding homologous regions in a conformation essential for cytotoxic activity. Such positioning, which could be achieved by a TNF disulphide bond, may require a very different sequence of amino acids in lymphotoxin, where no disulphide bond exists. These apparently unrelated regions of TNF and lymphotoxin might specify also as yet undiscovered differences in biological activities and/or target sites between the two molecules. The availability of efficient expression systems for TNF and lymphotoxin<sup>13</sup>, in combination with the techniques of site-directed mutagenesis<sup>40</sup>, will make it possible to address questions of this type directly.

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# Tissue-specific generation of two preprotachykinin mRNAs from one gene by alternative RNA splicing

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*A novel mammalian neuropeptide, the tachykinin substance K, is specified by a discrete genomic segment. Alternative RNA splicing generates two distinct mRNAs encoding the neuropeptide substance P alone or with substance K from a single preprotachykinin gene. Relative amounts of the mRNAs vary in different tissues, suggesting that the substance K-encoding sequence is regulated in a tissue-specific manner.*

SUBSTANCE P is one of the best characterized neuropeptides in mammalian tissues; several lines of evidence suggest that it acts as a neurotransmitter or neuromodulator in primary sensory neurones<sup>1</sup>. Substance P belongs to a family of related peptides, the tachykinins, and is thought to be the only member of this family present in mammalian tissues<sup>2</sup>. Recently, we elucidated the entire primary structures of two types of bovine brain substance P precursors ( $\alpha$ - and  $\beta$ -preprotachykinins) by determining their cloned cDNA sequences<sup>3</sup>.  $\beta$ -Preprotachykinin ( $\beta$ -PPT) contains not only the substance P sequence but also a novel tachykinin sequence designated substance K, whereas  $\alpha$ -preprotachykinin ( $\alpha$ -PPT) lacks the latter sequence, containing only substance P. The decapeptide substance K has been found independently as neurokinin  $\alpha$ , a gut-contracting peptide in porcine spinal cord<sup>4</sup>. The chemically synthesized substance K peptide possesses biological activities characteristic of the tachykinin family, but is considerably more potent than substance P in some pharmacological tests<sup>5,6</sup>. Substance K thus represents a second type of mammalian tachykinin which may have a physiological role different from that of substance P in mammalian organisms.

The two PPT mRNAs exhibit an interesting structural relationship. They have complete identity in their 5' and 3' sequences and differ only in the insertion/deletion of the sequence coding for the substance K region<sup>3</sup>. This characteristic structural relationship poses intriguing questions about the gene organization for these two mRNAs and the regulation for the generation of the two biologically different mammalian tachykinins. Our present investigations thus concern the structural organization of the PPT gene and the distribution and regulation of the two PPT mRNAs in the nervous system and peripheral tissues. We report here that the sequence specifying the substance K region is encoded by a discrete genomic segment, and that both  $\alpha$ - and  $\beta$ -PPT mRNAs arise from a single gene by alternative RNA splicing events. We also present evidence indicating the tissue-specific regulation of the PPT gene for the differential generation of the two PPT mRNAs.

## PPT gene organization

Genomic clones containing the bovine preprotachykinin gene were isolated from a bovine genomic library by hybridization

*in situ* with a bovine  $\beta$ -PPT cDNA probe, and all the isolated genomic DNA fragments were arranged into an approximately 36 kilobase-pair (kbp) length of a continuous genomic DNA (Fig. 1a; see Fig. 1 legend for experimental details of cloning). Nucleotide sequence analysis was performed on DNA fragments containing exons and their surrounding regions (Fig. 1b-f). Comparison of the genomic DNA sequence with the cDNA sequence enabled us to construct a structural organization of the bovine PPT gene (Fig. 1g). Intron A (403 base pairs, bp) is inserted within the segment encoding the 5'-untranslated region of the mRNA, 9-10 bp upstream from the translational initiation site. Introns B (~1.0 kbp), C (~450 bp), D (~460 bp), E (~1.4 kbp) and F (~3.6 kbp) all interrupt the protein-encoding region of the gene. The sequences at the exon-intron boundaries are consistent with the splice junction sequences observed for other genes<sup>7</sup>. Exons 2-7 consist of 132, 97, 45, 24, 54 and 596 bp, each encoding the protein sequence corresponding to the signal peptide, substance P, two spacer sequences, substance K, and the C-terminal sequence, respectively. It is remarkable that exon 6 precisely specifies the substance K region missing in  $\alpha$ -PPT. Because blot-hybridization analysis of total cellular DNAs (data not shown) as well as the genomic cloning described above showed that no more than one PPT gene is present in the bovine genome, we conclude that both  $\alpha$ - and  $\beta$ -PPT mRNAs are produced from a single gene as a consequence of alternative RNA splicing events.

The 5' termini of the PPT mRNAs were identified by S<sub>1</sub> nuclease mapping and primer extension analyses (Fig. 2). Both analyses revealed a length heterogeneity at the 5' end of the PPT transcripts. The major 5' termini of the PPT mRNAs mapped at 106-108, 110 and 111 bp upstream from the 3' end of exon 1 (Fig. 1g). Several minor mRNA species starting further upstream were also observed and these 5' termini mapped at roughly 132, 133, 137 and 146 bp upstream from the 3' end of exon 1. In support of these assignments, we found that three of the four cDNA clones isolated previously<sup>3</sup> (clones pSP301, 302 and 306) contained the extreme 5' sequences corresponding to the major 5' ends, while the remaining one (clone pSP307) extended its 5'-terminus up to one of the minor 5' ends. Based on the assignments of the 5' termini of the PPT mRNAs, we conclude that the bovine PPT gene is ~8.4 kbp long.